SPONTANEOUS AND INDUCED VARIATIONS IN BACTERIA.

A Thesis submitted to the University of Glasgow for the Degree of Doctor of Philosophy in the Faculty of Science.

by

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May, 1948.

ACKNOWLEDGMENT.

I wish to express my appreciation of the facilities accorded to me by the Council of the Hannah Dairy Research Institute, Kirkhill, Ayr, and the Department of Genetics, the University of Glasgow. My grateful thanks are also due to Dr N.C. Wright, Dr J.A.B. Smith, and Dr C. Higginbottom and other members of the Institute staff and particularly to Dr G. Pontecorvo of the Department of Genetics, the University of Glasgow, for their constant interest and valuable advice.

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S Y N O P S I S.

SPONTANEOUS AND INDUCED VARIATIONS IN BACTERIA.

Part I. Spontaneous and induced variations in bacteria.

Two different organisms, Bacterium prodigiosum and Bacterium aerogenes have been examined for Spontaneous variation. Individual colonies of these organisms were tested for their ability to grow in a simple, chemically defined medium in which the parent strain grows. Colonies which would not grow in this medium were taken to be of variant strains. Bact. aerogenes was employed for further work on induced variations or mutations as it gave a low rate of spontaneous variation.

Mutations were induced by X-rays and were conducted first by irradiation of broth cultures and later of bacteria in the dry state. Production of mutations in dried bacteria has not been reported previously. Different methods of suspension of the bacteria for drying, and different methods of storage were explored. After irradiation the cultures were examined for mutant strains using the method of Lederberg and Tatum. A critical study of this method was undertaken.

Two categories of mutants described as "adaptable" and "non-adaptable" were obtained. About 70% of the mutant strains were identified as unable to carry out the biosyntheses of certain amino-acids, vitamins,

purines or pyrimidines. The rest grew only with the addition of yeast extract to the medium. Some of the strains were further studied by investigating their response to related substance or substances which might be intermediates in the biosynthesis of the supplement required.

Part II. Studies on adaptations.

Further studies have been conducted on the "adaptable" strains. Variations occurring in these strains were examined but no conclusion could be drawn as to whether this was due to natural selection or mutation. Adaptation of these strains in different adapting media in the presence or absence of cell multiplication were studied. The only tentative conclusion that could be drawn was that no adaptation occurred in washed suspensions in the presence of a medium containing phosphate buffer and ammonium sulphate.

Part III. The histidine-less and nicotinic-less mutants of Bact. aerogenes A10α and the possibility of using them in microbiological assays.

The suitability of the simple medium for the parent strain was first studied from the point of view of maximum growth and acid production and various

modifications employed. Studies on the histidine-less and nicotinic-less mutants were then conducted by using the modified medium. The response of the histidine-less mutant (506, 297 and $9B_2$) to graded doses of histidine was found to be quantitative. The nicotinic-less mutants (893 and $3A_3$) responded to nicotinic acid in a peculiar way in that a "lag" in growth was found to occur at lower concentrations while no such "lag" occurred with nicotinamide. The effect of size of inoculum, the period of incubation, the H-ion concentration and the addition of a small amount of nicotinamide to the basal medium on the "lag" in growth at lower concentrations of nicotinic acid was then studied. Each of these factors were found to decrease the "lag" period completely or to a great extent.

GENERAL INTRODUCTION.

Within recent years the emphasis in genetics has been on the nature of gene action in development and function and in particular on the mechanism and the control of biochemical reactions by genes: this study has been termed biochemical genetics. Many interesting biochemical reactions in plants, animals and man can now be referred to the influence of specific genes - formation of antigens, flower and animal pigmentations, specific abnormalities in phenyl-alanine-tyrosine metabolism in man, mating reactions in protista, sugar fermentation by yeasts, atrophine esterase activity in rabbits and many others. These have been reviewed extensively by Wright (1941, 1945), Lawrence and Price (1940), Haldane (1938, 1942), Sonneborn (1942), Ephrussi (1942) and Beadle (1945). These studies have led to the suggestion that genes function in directing biochemical reactions with enzymes as intermediates between gene and reaction. (Beadle, 1945; Spiegelman, 1946; Muller, 1947).

The natural differences in the biochemical reactions mentioned above arise from gene loss or inactivation by mutation. Many of the strains among species of bacteria and fungi differentiated only by their different biochemical responses can also be considered to arise from gene loss or inactivation by mutation. A detailed and critical study of these and the variants

that may arise within each strain by mutation should give an insight into the biochemistry of some vital cellular reactions. Assuming that genes are in control of biochemical reactions it is of great interest to study the effect on these reactions of the loss or inactivation of genes by artifical methods. Mutations may be induced in a suitable organism and the morphological and biochemical characteristics of the mutant studied. It should be possible to convert an organism which originally synthesised a certain vital cellular constituent into one which now cannot do so, because of the loss or inactivation of the particular gene in control of this synthesis. Some reactions are obviously more easy to study than others such as the syntheses of amino acids, vitamins, purines and pyrimidines.

The study of these reactions necessitates the choice of an organism whose growth requirements are entirely under the control of the investigator. This rules out immediately animals as these cannot be grown on chemically defined media. The micro-organisms may be grown on such media but if genetic studies are to be made the choice would be further restricted to species whose genetic system is known. Certain bacteria of types intermediate between bacteria and fungi are not excluded.

The first investigation into the correlations

of the function of genes with biosynthesis in micro-organisms were made by Beadle and co-workers with Neurospora, the red bread mould, the genetics of which had been extensively studied by Shear and Dodge (1927) and by Lindegren (1942). Beadle and Tatum (1941) induced mutations in Neurospora, studied the genetic differences by crossing with the wild type of the opposite sex, and analysed the ascospores. They concluded that each mutation involved a single gene loss or change in a single gene. The method of identifying these mutant strains and studies of the specific reactions which are blocked have been described by Beadle and Tatum (1941); Horowitz, Bonner, Mitchell, Tatum and Beadle (1945); Tatum and Beadle (1942a) and Tatum (1944).

The principle underlying the method of identification of mutant strains is as follows: If a micro-organism capable of growth in a simple chemically-defined medium is caused to mutate with a consequent loss of synthetic power this micro-organism will not grow in the same simple medium unless the particular constituent which cannot now be synthesised is supplied. If after treatment different strains, first grown on a medium which supplies all the essential nutrients, are tested for their ability to grow on the synthetic medium containing only carbohydrate, an inorganic source of nitrogen, mineral salts, and, for Neurospora biotin,

those which do not grow unless some one essential nutrilite is added are assumed to be mutants.

A large number of Neurospora spores have been so treated and the mutant strains isolated. Most of the blocks have occurred in the synthesis of amino acids, vitamins or nucleic acid components. Strains which cannot reduce nitrate to nitrite, strains which fail to utilise fatty acids as a source of energy and those which can utilise saturated fatty acids but not their unsaturated analogues have also been obtained. Strains requiring sulphonamides have also been encountered (Emerson, 1947).

Subsequently, the same type of study was made by different workers on other fungi-Penicillium (Pontecorvo and Gemmell, 1944; and Bonner, 1946a), Ophiostoma (Fries, 1945), Aspergillus (Pontecorvo (1946, 1947); Diller, Tytell and Kersten, 1947; and Hocken-Hull, 1948), Absidia glauca (Giles, 1946), Actinomyces flaveolus (Kelner, 1947), and Saccharomyces cerevisiae (Tatum and Reaum, mentioned by Lindegren and Lindegren, 1947). Only with some of these has it been possible to show that each block in the synthesis of a vital cellular constituent means the loss or change of a gene, as the others are "imperfect" fungi.

No comparable studies correlating genes and biochemical reactions could be made with bacteria as these were held to possess no apparent sexual mechanism.

But the relative stability of the heritable growth factor requirements of many species of bacteria, e.g. Lactobacilli, suggest that they too possess a heredity-determining unit similar to that of the Neurospora and some other fungi and are equally capable of mutation. Some indication of the possession of such a unit was also given by the cytological studies of Robinow (1942, 1944) on different bacteria. Whatever the mechanism underlying mutation in bacteria, mutant strains are certainly useful tools because mutants requiring different nutrilites may be used in different studies as in microbiological assays and, more important, for the study of biosyntheses of vital cellular constituents, etc.

Roepke, Libby and Small (1944) and Gray and Tatum (1944) irradiated cultures of Escherichia coli with X-rays and obtained strains requiring specific nutrilites. Gray and Tatum (1944) also isolated biochemical mutant strains of Acetobacter melanogenum. Roepke, Libby and Small at the same time obtained similar strains from unirradiated cultures and were unable to establish any significant effect of the X-ray treatment. Further investigations of Tatum (1945) and Roepke (1946) established that increases in the rate of mutation occurred with X-ray treatment. A variety of deficient strains requiring amino-acids, vitamins, purines and pyrimidines were produced at random similar to the

mutants in other micro-organisms. That these mutations were produced independently of one another was shown by the fact that mutants with double deficiencies were obtained by re-irradiation of strains with single deficiencies. Triple mutants also were obtained by Lederberg (first mentioned by Tatum, 1946). These facts have led to the suggestion that bacteria possess genes or a similar heredity mechanism.

Recently after the present work began mutations have been induced in Eberthella typhosa (Grainger, 1947), Bacillus subtilis (Burkholder and Giles, 1947) and Staphylococcus aureus (Stone, Wyss, and Haas, 1947). No biochemically deficient strains were obtained from Eberthella typhosa but such strains, similar to those of E. coli, were obtained from Bacillus subtilis and Staphylococcus aureus.

Methods of production of mutations.

Different mutagenic agents have been employed for the production of these biochemical mutants. Beadle and Tatum (1941) used X-rays, neutrons, and ultra-violet light for the induction of mutations in **Neurospora.** X-irradiation has been used with Aspergillus, Penicillium, Ophiostoma, Actinomyces flaveolus, E. coli, Acetobacter melanogenum and Bacillus subtilis. Ultra-violet treatment has produced mutants with Absidia glauca, E. coli (Tatum

and Lederberg, (1947) and Bacillus subtilis. Ultra-violet light was less lethal than X-rays but Burkholder and Giles (1947) found that the percentage of mutants obtained with X-rays was double that obtained by ultra-violet treatment. Recently, Stone, Wyss and Hass (1947) produced penicillin-resistant and streptomycin-resistant strains of Staphylococcus aureus by previous ultra-violet irradiation of the nutrient broth into which the organism was later introduced. Mutation was produced by the same authors by irradiation of amino-acids and vitamins or of amino-acids alone before incorporating them in the medium.

Chemical agents have been found to be equally successful in inducing mutations. Horowitz, Houlahan, Hungate and Wright (1946) produced biochemical mutations of Neurospora using mustard gas. Mustard gas was also used for production of mutations in Aspergillus nidulans (Hockenhull, 1948) and Saccharomyces cerevisiae (Tatum and Reaum, mentioned by Lindegren and Lindegren, 1947). Tatum used nitrogen mustard (di-B-chloro-ethylmethylamine) as a mutagenic agent for the production of mutations in Neurospora (see Bonner, 1946b) and E. coli (Tatum, 1946). Recently, Wyss, Stone, and Clark, (1947) showed that as with ultra-violet irradiation, chemical treatment of nutrient broth with hydrogen peroxide and nitrogen mustard and subsequent incubation of Staphylococcus aureus

in the treated medium produced mutations. The same effect was produced by similar treatment of solutions of certain amino acids, purines and pyrimidines before addition of the nutrient broth. So far these different methods of treatment have produced the same types of biochemical mutants. Perhaps genetic investigations may show some new genotypes among those obtained by chemical treatments.

In the case of fungi, conidia both dry and in saline suspension have been exposed to mutagenic agents (Beadle and Tatum, 1941; Kelner, 1947). Cultures of bacteria have been exposed either in nutrient medium (Gray and Tatum, 1944) or, after centrifuging and washing, in saline suspension (Grainger, 1947). Burkholder and Giles (1947) have irradiated the spores of Bacillus subtilis suspended in their "complete" medium. For treatment with nitrogen and sulphur mustard the organisms were suspended in buffer solutions and the chemicals added in the required amount. Stone, Wyss and Hass (1947) and Wyss, Stone and Clark (1947) exposed the broth or the chemical solutions and then added the organism, incubated the culture and examined for the mutants.

Suitable dilutions of the exposed organisms were plated on a rich medium which was presumed to contain all the necessary nutrients. Well-isolated colonies were then picked to agar slopes of the rich medium and

each colony then tested for the ability to grow in a simple synthetic medium ("minimal") in which the unexposed organism would grow. Any strain which did not grow in this medium was taken to be a mutant strain. Lederberg and Tatum developed another technique for the isolation of mutants by which, first, the unmutated cells were allowed to grow on "minimal" agar medium and, then the mutated cells by the addition of a rich medium.

There are certain obvious disadvantages in this method. The rich medium employed contains yeast extract which is well known to contain certain inhibitory factors and which may kill off some strains. Testing in one synthetic medium under one set of conditions - pH and temperature - involves the risk of losing possible mutant strains as some of them grow normally only at certain H-ion concentrations and temperatures, for example in Neurospora the pyrodixine-less mutant (Stokes, Foster and Woodward, 1943), riboflavine-less mutant (Nitchell and Houlahan, 1946a), and the adenine-less mutant (Mitchell and Houlahan, 1946b) and in Aspergillus the adenine-less mutant. A possibly better method of selection of mutants would be to plate on a series of rich media and then test the growth of each colony in a series of synthetic media at different temperatures, different H-ion concentrations, different oxygen tensions, etc. This would involve

Herculean labours and Penelope-like patience.

The mutation rate, with X-rays, varies with the dosage i.e. the higher the dosage the greater the mutation rate (Sansome, Demerec and Hollaender, 1945; Grainger, 1947; Roepke and Mercer, 1947). It is also found to vary with the age of the organism treated. Young conidia of Neurospora (2 to 3 days old) are more sensitive to nitrogen mustard treatment than older ones (7 to 12 days old) (Miller and McElroy, 1948). The efficiency of mutation with ultra-violet radiation varies with the wave length in the same way as its absorption by nucleic acid suggesting that the energy effective in producing mutations in genes is absorbed by nucleic acid. (Hollander and Emmons, 1941; Stadler and Uber, 1942).

The identification of mutants involves the addition of known single compounds to the synthetic media inoculated with the mutant strains. If growth is obtained with any particular compound that compound is taken to be the nutrient required by that strain. A simplified method devised by Beijerinck in 1889 has been systematised by Pontecorvo (1947).

Biochemical and biological applications of the mutants.

Mutant strains of micro-organisms have been used in various ways in biochemical and biological studies.

One of the most fascinating fileds in biochemistry is that of the biosyntheses of vital cellular constituents.

That the cell is able to synthesise a series of compounds which the chemist is unable to synthesise has been known for several years. Only a few instances are known in which the chemist can describe in detail the chain of reactions involved in the biosynthesis of compounds like amino-acids and vitamins. This lack of knowledge is partly due to the instability of the intermediate products. The use of radio-active and other isotopes as tracer elements have brought about great advances but this method has a disadvantage in that there is no way to stop the chain of reactions at each step and to find out the chemical nature of the intermediate products. Biochemical mutations of micro-organisms have supplied some useful material for such studies. Mutants have been obtained in which a sequential series of reactions have been blocked at different stages. Biochemical investigations with certain of these mutants have shown so far the paths of syntheses followed in micro-organisms of certain B-vitamins and amino-acids. Srb and Horowitz (1944) have analysed fifteen mutant strains of Neurospora requiring arginine and shown that the ornithine cycle present in mammalian liver is also present in this mould. The same series of reactions have been demonstrated in Penicillium, (Bonner, 1946b) and E. coli (Roepke, 1946). It is doubtful if this similarity between mammals and micro-organisms could have been

demonstrated by any other method. The biosynthesis of tryptophan through anthranilic acid, indole and serine has been shown for Neurospora (Tatum and Bonner, 1944; Tatum, Bonner and Beadle, 1944) and for E. coli (Roepke, 1946) and Asperigillus nidulans (Pontecorvo, unpublished). The syntheses of cystine and methionine from sulphates through the stages of sulphite, sulphide, cystine, homosysteine, cystathionine to methionine in Neurospora, Aspergillus, Ophiostoma and E. coli have been established by different workers (Fries, 1946b; Horowitz, 1947; Lampen, Roepke, and Jones, 1947; Pontecorvo, unpublished). The biosynthesis of aneurin through thioformamide and thiazole have been demonstrated in Neurospora (Tatum and Bell,1946) and in Aspergillus (Pontecorvo, unpublished). Choline synthesis has been demonstrated through stages of aminoethanol, monomethyl aminoethanol, dimethyl aminoethanol to choline (Horowitz, 1946; Horowitz, Bonner and Houlahan, 1945). Nicotinic acid has been shown to be formed in Neurospora (Bonner, and Beadle, 1946; Beadle, Mitchell and Nye, (1947); Mitchell and Nye, 1948; Bonner, 1948) and in Aspergillus (Pontecorvo, unpublished) from anthranilic acid, indole, tryptophan, kynurenine, oxypyridine carboxylic acids, and hydroxy anthranilic acid.

Certain inter-relationships between amino-acids have been established by biochemical studies of the mutant strains. That iso-leucine and valine are

interrelated has been shown by studies on Neurospora and E. coli (Bonner, Tatum and Beadle, 1943; Bonner, 1946a; Bonner, 1946b; Tatum, 1946). The interrelation of serine and glycine (Hungate, 1946; Gray and Tatum, 1944; Horowitz, Houlahan, Hungate and Wright, 1946), and of proline, glutamic acid and glutamine in Neurospora and E. coli (Bonner, 1946b; and Tatum, 1945), of phenyl-alanine and tyrosine (and the possibility that tyrosine is not synthesised from phenyl-alanine) in E. coli, (Simmonds, Tatum and Fruton, 1946), of lysine and arginine in Neurospora (Doermann, 1945) and in Aspergillus (Pontecorvo, unpublished), of citrulline and uracil in E. coli (Roepke, 1947) and of various other vitamins and amino acids have been demonstrated. The multiple deficient mutants of Bacillus subtilis obtained by Burkholder and Giles (1947) -valine, isoleucine, and phenylalanine; proline and cystine; methionine and threonine; histidine and leucine - may be of great interest. These studies have also shown that the biosyntheses of amino-acids do not always follow the path of reductive amination of keto-acid analogues. Thus a new and very wide field of study has arisen in biochemistry based on the investigation of these mutants.

The mutant strains can be used with great advantage in microbiological assays. Those mutants which respond to one specific substance can be used in bio-assays of that substance provided the response is quantitative.

Such assays have been developed among others for pyridoxine (Stokes, Larsen, Woodward and Foster, 1943), p-aminobenzoic acid (Thompson, Isbell and Mitchell, 1943), leucine (Ryan and Brand, 1944), lysine (Doermann, 1945) and choline (Horowitz and Beadle, 1943). Assays with E. coli mutants have also been suggested (Lampen, Jones and Roepke, 1944). The advantages are that a comparatively simple medium can be used, the preparation of which invloves less labour and less expense than that of the complicated medium required by Lactobacilli, etc., and that a choice of different strains of one and the same micro-organism is available.

Certain mutants have been obtained with E. coli where phage resistance can be correlated with nutrilite requirements for tryptophan, for proline and for some other unidentified substances (Anderson, 1944, 1946; Wollman, 1946). These associations have been proved to be multiple effects of the same mutations. Such mutations indicate the possibility of interpreting phage resistance in terms of specific metabolic changes, and hence of deriving information on the biochemistry of phage growth.

Mutation instead of blocking or retarding may also accelerate a reaction. Industry would greatly benefit if mutant strains of industrially important micro-organisms with increased production of the desired end-product could be obtained e.g. mutant

strains of Bact. aerogenes which is used in the production of acetyl-methyl carbinol.

Studies with multiple mutants have shown that gene recombinations can take place in bacteria too, and are discussed in detail by Tatum and Lederberg (1947). Lederberg (1947) has found evidence for a linear order of genes in E. coli.

Studies in syntrophism with the different mutant strains requiring the same end-product throw some light on symbiosis as do studies with related strains of different species of bacteria and fungi occurring in nature. These related strains produce some nutrilites which can be used by others and so these strains in the course of evolution develop a tendency to grow together and the strains become symbiotic.

Drug and antibiotic resistant strains of Neurospora and E. coli are known and enzymatic studies with these will no doubt throw some light on the competition - inhibition phenomena observed with many cellular constituents.

The classifications of bacteria in current use are determinative keys intended for practical use by certain groups of specialised workers and emphasise the category of bacterial similarities or differences in which these workers are interested. Thus many bacterial species and genera are often separated on the basis of character differences that may be brought

about by a single mutational step. For example, the genus Phytomonas are separated from the closely related genus Pseudomonas on the basis of plant pathogenicity which may well arise or disappear by mutation. Another system of classification can be established by a more fundamental differential criterion involving a large number of differences in independently variable morphological and biochemical characters. A complex metabolic process involving elaborate chains of reactions should be a valuable taxonomic criterion provided the differences between phenotypes cannot be traced to a change in one single link in the reaction. Thus a study of artificial mutations would throw light on how far the differences in phenotypes are due to a single mutation or to a series of mutations involving an entirely new path of biosynthesis which would justify classification into a new genus or species.

The present investigation was undertaken with the object of obtaining mutant strains of bacteria and of studying the biochemical characteristics of these mutants in order to throw some light on the biosyntheses of vital cellular constituents and on the possibilities of their use for microbiological assays.

PART 1.

SPONTANEOUS AND INDUCED VARIATIONS IN BACTERIA.

INTRODUCTION.

In the work presented here, two different organisms, Bact. prodigiosum and Bact. aerogenes, were examined for spontaneous and induced variations. Only variants or mutants induced by X-rays were investigated. One distinctive feature of this work was that the mutations were produced by treatment of the bacteria in the dry state. Other workers (Roepke, Libby and Small, 1944; and Gray and Tatum, 1944; and Burkholder and Giles, 1947) have treated the bacteria or the spores in liquid suspension. The fact that mutations can be produced in dry bacteria shows that active growth and division is not an essential condition for the induction of mutations in bacteria. The induction of mutations in spore suspensions of Bacillus Subtilis by X-rays and ultra-violet light supports this view (Burkholder and Giles, 1947).

A new similarity has therefore been shown between mutation in bacteria and in higher micro-organisms, since in the latter non-dividing and relatively quiescent cells such as spores and spermatozoa are known to mutate readily under the action of mutagenic agents. The examination for mutants in irradiated dry bacteria was conducted soon after irradiation and not some hours later as in the technique of other

workers who used bacterial or spore suspensions (Roepke Libby and Small, 1944; Gray and Tatum, 1944; Burkholder, and Giles, 1947; Stone 1947 and others). The dosage required to produce a satisfactory mutation rate 180,000 roentgen units was of the same order as that used by Gray and Tatum (1944) with E. coli. These authors obtained after irradiation a survival of 0.01% while in the present work a survival of 0.01 to 0.03% was obtained. The mutation rate at this dosage obtained by Gray and Tatum was 0.25% for E. coli and 0.6% for Acetobacter melanogenum. The mutation rate for Bact. aerogenes A10c varied from 0.1-1.0%.

Two different kinds of mutants have been obtained, "adaptable" and "non-adaptable". The "adaptable" mutants were those showing delayed growth in "minimal" medium without addition of any other nutrilite and "non-adaptable" were those which did not grow at all without addition of a nutrilite. The "adaptable" mutants were obtained only from irradiated bacteria. These mutant types may, perhaps, fall in the same category as those mutants of Neurospora which require adenine, riboflavine or pyridoxine only at certain pH and temperatures (Mitchell and Houlahan, 1946; Stokes, Foster and Woodward, 1943); and the adenine-less mutant of Aspergillus nidulans which requires the nutrilite only at certain temperatures (Pontecorvo, unpublished.)

Most of the mutants obtained have been characterised in more detail in order to find out how far they could be used in microbiological assays and also to find out at what stage the block in the biosynthesis of that particular nutrilite took place. One of the important criterions of a suitable organism for a microbiological assay is that the organism should respond only to one particular nutrilite and not to related or breakdown products of the nutrilite. For example, an aneurin-requiring organism should not respond to 4-methyl-5-β-hydroxy-ethyl thiazole and 2-methyl-4-amino-5-ethoxy-methyl pyrimidine nor should a leucine-requiring organism respond to either iso-leucine or nor-leucine. These detailed biochemical studies have shown that a few of the mutants satisfied this particular criterion. They have also confirmed in a few instances some part of the sequence of reactions in the biosyntheses of nutrilites as put forward by other workers.

SECTION 1.

SPONTANEOUS VARIATION OR MUTATION IN BACTERIA.

In the choice of a micro-organism for the present investigation certain criteria were used. It should be able to grow in a simple synthetic medium, containing an inorganic source of nitrogen, produce a reasonable amount of acid, have a low spontaneous mutation rate,

have no tendency to grow in clumps, be non-motile and preferebly pigmented as the colour or lack of colour will help in distinguishing any change in colony appearance.

As an organism which might satisfy some at least of these criteria, especially that of pigment production, attention was first directed to Bacterium prodigiosum (Serratia marcescens).

Bact. prodigiosum (National Collection of Type Cultures No. 2302) is a rod-shaped organism, gram negative, motile and forms a red pigment. The optimum temperature for the development of the red colour in 24 hours was found to be between 22 to 30°C. and so all experiments with this organism were conducted at 25°C. It produced acid but no gas in glucose, sucrose, mannitol and maltose, no acid or gas in lactose. There was slight acidity in litmus milk in 24 hours - acid and clot in 3 days. It gave positive methyl red and Voges-Proskauer tests, reduced nitrate to nitrite, did not form indole and liquefied gelatine.

The growth of this organism in three different synthetic media was tested. (1) Hucker's medium with added magnesium sulphate (Hucker, 1924) (2) Dewey and Poe's synthetic medium for Serratia (Dewey and Poe, 1943) (3) Lodge and Hinshelwood's medium for Bact. lactic aerogenes (Lodge and Hinshelwood, 1943.) The

organism grew well in media (2) and (3). Medium (3) was chosen as it was simpler than (2).

This strain of Bact. prodigiosum was then purified by plating suitable dilutions of a 24 hour culture (25°C) in standard broth with standard agar. After two days incubation at 25°C, the different coloured colonies were picked and were designated as red, pink and white types. The white type on two replatings gave only white colonies, the red and pink gave all three types on replating. The pink colonies usually turned deeper in colour on continued incubation of the plates. The red type was replated again and again and on fifth and sixth replating gave mostly red and a very few pink colonies which turned red after three days incubation. The red type strain from the sixth plating was thus considered to be apparently stable. Bunting[1940] has shown that Serratia marcescens, strain 274 (Bact. prodigiosum) grown in a well buffered ammonium citrate-glycerol medium consisted of four types of colonies; dark red, bright pink, pale pink and white. Each type on replating gave one or more variant types, equilibrium between types being attained rapidly in growing cultures. Thus Bunting with her media was not able to get any stable type. The red type isolated in the laboratory was apparently stable in standard agar medium but when this strain after single cell isolation was later grown on "complete" agar medium it gave again the three different types.

Thus, it appears that the stability of the strain depends on the nutrient medium.

The culture from a red cell isolated from the sixth plating (as described above) was used for single cell isolation, following Gardner's modification of Ørskov's method (Stoughton, 1931).

SPONTANEOUS VARIATION IN BACT. PRODIGIOSUM.

The single cell culture as obtained above was examined for spontaneous variants or mutants. The method employed was to grow well-isolated surface colonies on a complete agar medium, transfer them to individual slants of the same agar and then to test their ability to grow in a "minimal" medium in which the parent strain grows well. This method has been called "total isolation method" by Fries, (1947).

The "complete" medium had the following composition.

Peptone	10.0 g.
Yeastrel	5.0 g.
Glucose	10.0 g.
Agar	15.0 g.
Tap water	1000 ml.

pH 7 to 7.2

The addition of lemco to the above medium did not improve growth to any appreciable extent. Peptone - lemco - yeast extract - agar with or without glucose

did not give as heavy a growth as the above.

"Minimal" medium.

The medium was that used by Lodge and Hinshelwood for Bact. lactic aerogenes. The composition was:-

Ammonium sulphate	1.0	g.
Potassium dihydrogen phosphate .	3.46	g.
Magnesium Sulphate ($MgSO_4$, $7H_2O$)	0.038	g.
Glucose	38.5	g.
Distilled water	1000	ml.

The pH was adjusted to 7.2 with sodium hydroxide.

The growth of the parent strain was found to be quite satisfactory in this medium. The acidity obtained in 10 ml. was equivalent to 8.35 ml. N/50 alkali. Modifications of this medium with changes in the source of nitrogen, in the amount of glucose, addition of salts, and changes in the pH were tested in relation to acid production.

The replacement of the source of nitrogen with nitrate instead of ammonium gave less growth.

The acidity produced in 5 ml. medium with 1, 2, 3, 4 and 5 percent glucose with and without salts was determined. A salt solution containing 0.7 g. KCl, 0.5 g. $FeSO_4$, $7H_2O$ and 0.5 g. $MnSO_4$, $4H_2O$ in 250 ml. was prepared and 5 ml. of this added to 1000 ml. of the "minimal" medium. The results are given in Table 1.

It can be seen from Table 1 that addition of

TABLE 1.

Acidity produced with differing amounts of glucose with and without salts.

Acidity produced in 48 hours at 25°C. 24 hour culture used as inoculum. 5 ml. of medium. Acidity expressed as ml. N/50 Sodium hydroxide.

Percentage of Glucose.	Ml. N/50 NaOH.	
	Without salts	With salts
1	4.0	3.9
2	4.0	4.1
3	4.1	4.2
4	4.2	4.2
5	4.2	4.3

TABLE 2.

Effect of change in pH of medium.

Acidity produced in 48 hours at 25°C. 24 hour culture used as inoculum. 5 ml. of medium. Acidity expressed as ml. N/50 Sodium hydroxide.

pH of medium.	Ml. N/50 NaOH.
6.0	3.7
6.4	4.0
6.8	4.2
7.2	4.4

salts did not bring about any significant change in the amount of acid produced and so this addition was considered unnecessary. The increase in the percentage of glucose from 1 to 5 brought about a change from 3.98 to 4.24 only. As this increase was not very striking 2% glucose in the medium was arbitrarily chosen.

Effect of changes in pH of the medium.

Growth in media at pH adjusted to 6.0, 6.4, 6.8, and 7.2 was tried. The results are expressed in Table 2 from which it can be seen that the optimum pH was about 7.

The composition of the "minimal" medium finally chosen was:-

Glucose	20.0 g.
Ammonium sulphate	0.96 g.
Potassium dihydrogen phosphate ..	3.46 g.
Magnesium Sulphate, $MgSO_4$, $7H_2O$.	0.04 g.
Distilled water	1000 ml.

pH 7 - 7.2.

Examination for variants or mutants.

0.05 ml. of suitable dilutions of 21 hour (25°C) broth cultures were spread on the surface of prepared plates of "complete" agar medium to give approximately 40 to 70 well-isolated colonies. After 24 hours at 25°C, colonies were picked to "complete" agar medium slopes. After 24 hours the culture from each slope was inoculated into "minimal" broth medium. Any

TABLE 3.

Results of the examination for spontaneous variation in Bacterium prodigiosum.

Expt. No.	No. of colonies examined.	Mutants obtained.	Experimental No. of the mutant strains	Colour of the mutants.
1.	228	0		
2.	599	8	311, 458, 542, 579, 607, 680, 742, 827.	All pink except 542(white)
3.	727	5	897, 967, 1063, 1184, 1242.	All pink.
Total.	1554	13		

subculture which did not grow within 24 hours in the "minimal" medium was considered to be a variant of a mutant.

Three experiments were conducted in which altogether 1554 colonies were examined and thirteen mutants obtained. It should be mentioned here that though the parent culture was from a single cell red type, plating for the examination of colonies again gave a mixture of red, pink and white cells. In the first experiment no mutant was found among the 228 colonies picked. In the second experiment, 8 mutants were found among the 599 colonies picked. Seven out of the eight were pink, the eighth was white. In the third experiment, five out of 727 colonies were mutants; all were pink. The results are brought together in Table 3. The colonies were numbered consecutively from one upwards.

Identification of the mutants.

These strains were first tested morphologically and biochemically and were found to behave like the parent strain except for the additional nutrient requirement. They were then tested in a systematic way by the method developed by Pontecorvo (1947), similar to the auxanographic method of Beijerinck, to find out what substance or substances these strains require in order to grow in "minimal" medium. A few crystals of test substances were placed on different

parts of prepared plates inoculated with the mutant strains. The test substance diffused into the surrounding medium and growth took place when the test substance could serve as a nutrilite for the mutant.

This is a better method of identification than that of the addition of substances to liquid medium when there is the possibility of their concentrations being not optimal, i.e. the concentrations may be either too low or too high causing inhibition of growth. Such unsuitable concentrations are avoided by the placing of crystals of test substances on solid medium. Even if the amount is too high on the spot where the crystals are placed zones in which the concentrations are optimal will occur in the surrounding medium.

This method has been simplified still further by Pontecorvo (personal communication) by testing first with complex substances like a vitamin-free casein hydrolysate instead of amino-acids, yeast extract, mixtures of vitamins, purines, pyrimidines or yeast and thymine nucleic acids. If growth occurs with any of these then growth with individual amino-acids, vitamins, purines or pyrimidines can be tested as the case may be. This simplification involves less labour and less wastage of some of the expensive and scarce chemicals.

The substances tested were as follows:-

Vitamin-free casein hydrolysate, Yeast extract,

Thymine nucleic acid.	Nicotinic acid.
Calcium -d-pantothenate.	Pyridoxine hydrochloride.
Aneurin hydrochloride.	Choline chloride.
P-Aminobenzoic acid.	Inositol.
Biotin.	Folic acid.
Riboflavin.	Glutamic acid.
L-Tryptophan.	L-Tyrosine.
L-Cystine.	DL-Serine.
L-Histidine monohydrochloride.	DL-Isoleucine.
D-Arginine monohydrochloride.	L-Leucine.
DL-β-phenylalanine.	DL-Valine.
DL-Lysine hydrochloride.	DL-Threonine.
DL-Methionine.	DL-Alanine.
Glycine.	Proline.
Hydroxy-proline.	Asparagine.
Uracil.	Uric Acid.
Xanthine.	Guanine.
Adenine.	Thymine.
Yeast nucleic acid.	Glutamine.
Aspartic acid.	

Method of testing.

A 24 hour culture in "complete" medium (broth) was centrifuged, washed twice with sterile $\frac{1}{4}$ strength Ringer's solution and a loopful of the third suspension on "minimal" agar medium plates and dried. A few crystals of the above substances (usually four different

TABLE 4.

Growth factor requirements of the mutant strains of Bact. prodigiosum.

Mutant.	Colour.	Growth factor requirements.
458	Pink.	p-Aminobenzoic acid.
827	"	p-Aminobenzoic acid.
311	"	p-Aminobenzoic acid or choline chloride. Growth with choline chloride was slower.
607	"	
579	"	
680	"	
542	White.	
742	Pink.	Grows well with choline chloride and slightly with leucine.
897	"	Methionine or cystine. Also cystein hydrochloride.
967	"	Glutamic acid. Asparagine gives good growth in 2 days. Slight growth with tryptophane, tyrosine and cystine.
1063	"	No growth with any of the single substances tried. Good growth with yeast extract and a slight growth with a mixture of all the vitamins.
1184	"	Tryptophane.
1242	"	Slight growth in two days with leucine, phenylalanine and valine. Combinations of these three acids did not give better growth.

ones on each plate) were put on the inoculated agar surface and the plates incubated at 25°C. As the culture would not grow in "minimal" agar medium alone a good confluent growth on any part of the plate was taken to indicate that the strain required the addition of that particular substance to the "minimal" medium in order to give good growth. This was confirmed in "minimal" medium (broth) to which the particular substance or substances were added. Where good growth was obtained the strain was considered to be a variant or mutant requiring that particular substance.

The growth factor requirements of the thirteen mutant strains are given in Table 4.

From the above results it can be seen that in Bacterium prodigiosum the spontaneous variation or mutation was very high, thirteen in 1554, that is the percentage was as high as 0.84%. Therefore, it was decided that this organism was not a suitable one to use in the present investigation.

Spontaneous variation in Bacterium aerogenes.

A few strains of coliform organisms isolated in the laboratory from various sources and designated as S, S_1, S_2 (from soil), D_1 (from dung), A10C, K7, X1B, G18A and LW6C (obtained from dried milk) were examined for their ability to grow in the "minimal" medium described above and for the amount of acid

TABLE 5.

Spontaneous variation in Bact. aerogenes A10c.

Expt. No.	No. of colonies picked.	No. of mutants obtained.	Substance required by the mutant.	Mutant No.
1	750	1	Not yet found. Grew well with yeast extract.	385
2	500	1	Nicotinic acid or amide.	893
3	685	0		
Total.	1935	2		

produced in the medium. Except for S_2 and X1B, the amount of acid produced was the same but some of the strains grew in clumps. Bact. Aerogenes A10C was considered to be the most suitable.

Bact. aerogenes A10C is a gram-negative, non-motile rod giving translucent, smooth colonies. It gave acid and gas in glucose and lactose, acid and gas in litmus milk which was completely decolourised in 3 days and a trace of peptonisation. It gave negative methyl red, positive Voges-Proskauer and citrate tests, liquefied gelatine and did not form indole. It grew vigorously in all the three synthetic media described on p.20 . Optimum growth was at 37°C.

This strain was purified by three platings and the culture obtained used for single cell isolation, following Gardner's modification of Ørskov's method.

Examination for variants or mutants.

The examination was conducted by the same methods as already described for Bacterium prodigiosum. Instead of a 24 hour culture, a 5 hour broth culture (37°C) was used.

The results of three experiments in which 1935 colonies were examined are given in Table 5 along with the growth factor requirements of the mutants. The colonies were numbered consecutively from one upwards.

The mutants were examined morphologically and

biochemically and found to be the same as the parent strain except for the additional nutritional requirement.

Mutant 385 did not grow with any of the substances tried and was further tested with the following substances or combination of substances; asparagine, asparagine + 10 vitamins, tyrosine, mixture of purines and pyrimidine - adenine, guanine and uracil, and the same mixture + 10 vitamins. With asparagine it showed some growth in two days, with asparagine and ten vitamins some growth in one day and good growth in two days. It grew well on addition of a drop of yeast extract and also in peptone water. It has not been possible to characterise further the nutritional requirements of this mutant.

Mutant 893 was plated three times choosing each time a colony which did not grow in "minimal" medium. The strain chosen from the third plating will be referred to as nicotinic-less mutant (893) of Bact. aerogenes A10C.

Since the rate of spontaneous mutatation of Bact. aerogenes A10C was only 0.1% (2 in 1935) and as it gave satisfactory growth in "minimal" medium with reasonable acid production, this micro-organism was considered to be satisfactory for the production of artificial mutants.

SECTION 2.

INDUCED MUTATIONS OF BACT. AEROGENES A1OC.

In the present investigation, mutations were induced by X-rays. Both broth and dried cultures were used. Viable counts of the cultures before and after irradiation gave an approximate estimation of the survival rate. The dosage was chosen to give a survival of one in 1000 to one in 10,000. After exposure the treated cells were plated and examined. At first, well-isolated single colonies were picked and each strain originating from each single colony isolation tested for their inability to grow in the "minimal" medium. This method was employed only when the broth cultures were being irradiated. Later, when the dried cultures were being irradiated the selective technique of Lederberg and Tatum was adopted. Both techniques were inevitably selective but the latter was more so. It is possible, as discussed later, that by this method many types of mutants are missed. It was adopted, however, because it involved much less labour and time than the former technique. The nutritional requirements of most of these mutants have been identified and in some cases the specificity of the response to various nutrilites tested.

Irradiation of broth cultures.

The organism derived from a single cell and at a

later date repurified by picking a single colony from "minimal" agar medium was subcultured four times serially in "complete" broth medium of the composition already given. 0.2 to 0.3 ml. of the fourth subculture (24 hour) was diluted 1:1 with sterile broth, and sealed in thin-walled glass tubes of 3 to 5 mm. diameter and 2 to 2.5 cm. long, ready for irradiation.

The apparatus used for irradiation was a Watson Mobilix X-ray unit with an oil-cooled Coolidge tube (tungsten target), inherent filtration equal to 2 mm. Al. It was operated at 80 kV and 5 mA. The dosage at a distance of 7.5 cm. from the target was 1500 roentgens per minute.

At first the tubes were held as near the target as possible but heat production was appreciable and, in long exposures, sufficient in itself to kill off the organisms. Later, the tubes were held at a greater distance from the target. The intensity and dosage of irradiation were varied by altering the distance from the target and the time of exposure. The tubes were shaken at 5-10 minutes intervals to ensure that all the micro-organisms were uniformally exposed.

Four irradiations of broth cultures, entirely of an exploratory nature, were conducted giving different doses at different intensities.

The broth cultures were prepared at the Hannah Institute and transported in an ice-cooled Dewar flask

TABLE 6.

Results of irradiation of broth cultures. (Bact aerogenes $A10_C$).

Exp. No.	Type of culture treated.	Viable count per ml. $x10^6$	Distance of the bacteria from target. cm.	X-ray irradiation. Time and dose in roentgens.	Viable count per ml. after irradiation.	No. of colonies picked.	No. of mutants obtained.	Mutant No.
1.	24 hour culture in complete medium, diluted 1:1	240	3.5	(1) 270,000-292,500 (½ hour)	540	none	nil.	
				(2) 63,500- 89,500 (1 hour)	$588x10^4$	282		
2.	" " " "	236	3.5	239,050 (1 hour)	$75x10^2$	582	3	(1) 297 (2) 506 (3) 518
3.	" " " "	2,600	3.5	235,250 (42 mins.)	$< 1x10^3$			
4.	" " " "	327	3.5	(1) 121,500 (20 mins.)	$100x10^3$	not picked.		
			7.5	(2) 15,000 (40 mins.)	$71x10^4$	not picked.		
			3.5	(3) 225,000 (40 mins.)	768	600	nil.	

to the Department of Genetics in Glasgow University where the irradiation took place. After irradiation cultures were replaced in the Dewar flask and taken back to the Hannah Institute. The irradiated cultures were then examined for mutant strains, not less than four hours after irradiation. The cultures were kept in the interval in the ice-cooled Dewar flask only.

The treated bacteria were transferred quantitatively to another tube washing with sterile ¼ strength Ringer's solution and the volume made up to 10 ml. Suitable dilutions of this were then spread on plates to obtain growth of individual colonies and the mutants isolated as described on p.24. The results are recorded in Table 6. In Experiment 2 following a dose of 239,050 r three mutants were detected in 582 colonies tested. In Experiment 3 with approximately the same dose no colonies were obtained on plates spread with aliquots of 1:1000 dilution of the exposed culture. In Experiment 4 colonies were picked only from the culture exposed to 225,000 r but no mutants were obtained in 600 colonies examined. No mutants could be obtained from cultures exposed to lower dosages.

The irradiation of broth cultures required very high dosages for any mutation to take place. Such high dosages could be obtained in a reasonable time (40 minutes to one hour) only by a high intensity of radiation. This in turn involved holding the tube close up to the target where there was every chance that the heat produced would contribute to the death of the bacteria. In view of the danger of over-heating and the difficulty of transportation the irradiation of

broth cultures was abandoned and attention was directed to cultures in the dry state.

Irradiation of cultures in the dry state.

Preparation of bacterial suspensions for drying.

Various methods of drying the bacterial suspensions were investigated in order to obtain a uniform distribution of the bacteria over a flat surface in a dry film which could be easily reconstituted and in which there would be a fair survival of bacteria after drying.

Different kinds of supports for the bacterial suspension were investigated. Filter paper and a thick cellophane were discarded. With the former there was difficulty in breaking up the paper and with the latter the supports after drying did not remain flat. The best support was considered to be small disks of thin permeable cellophane 600 .

It is well known and has been confirmed in a recent paper by Stamp (1947) that the medium in which the bacteria are suspended affects their survival after drying. In the present investigation, suspension in sterile $\frac{1}{4}$ strength Ringer's solution or sterile water gave survivals below 1%. A dilute solution of the complete medium (1:1) gave a fairly satisfactory survival of 33.5% on the average.

The methods adopted to obtain a uniform distribution on the disks were as follows:-

(a) The disk was dipped in "complete" broth medium

before drying. This method had to be discarded since the bacterial suspension was on both sides of the disks with the result that part of it stuck to the container during drying (see below) and consequently no uniform preparation could be obtained.

(b) Growth was allowed to spread over permeable cellophane disks of uniform area placed on the surface of inoculated agar plates. The "complete" agar medium surface was flooded with a 24 hour culture (third subculture), the excess removed by a sterile pipette and the agar surface allowed to dry. The sterile disks were put on the dried surface, the plates were incubated at 37°C and the disks then removed and dried. This method too was discarded because the growth was not uniform and also because part of the growth stuck to the bottom of the dish as in (a).

(c) A thick suspension of the bacteria was prepared by centrifuging a broth culture and resuspending in a smaller volume of diluted sterile broth. Of this suspension 0.01 ml. was spread over the cellophane disks and dried. A heavy and fairly uniform preparation was obtained by this method.

The method of suspension finally adopted was as follows: a fourth subculture on "complete" agar medium slopes (24 hour 37°C) was suspended in dilute "complete" broth medium and centrifuged for a minute at a low speed to bring down any clumps. The turbidity of the

supernatant suspension was adjusted to a Spekker reading of 1.75 to 1.85 and a viable count of approximately 12 to 14 x 10^7. Of this suspension 0.01 ml. was measured from a pipette to disks of permeable cellophane 600. (approximately 1 cm. in diameter). The disks were first sterilised by autoclaving after placing them flat and separate between folds of filter paper in a petri dish. The preparations were then dried.

Method of drying.

The method followed was that of the lyophile process applied by Nyman, Gunsalus and Gortner (1945) to the maintenance of cultures for microbiological assay. The disks as prepared above were put in sterile petri dishes and cooled in a refrigerator. After cooling for about one hour, the petri dishes were put in a cooled vacuum desiccator with anhydrous calcium chloride as the drying agent. The desiccator was attached to a Cenco Hyvac pump, the pressure reduced quickly and maintained below 1 mm. of mercury for about 2 hours. The desiccator was then disconnected from the pump and the cultures kept overnight under vacuum. This gave a preparation which could be reconstituted fairly easily provided the cooling and evacuation was done rapidly and completely.

The disks after drying were covered with larger cellophane disks in order that the preparations might

TABLE 8.

Survival of dried bacteria at room temperature without vacuum.

Experiment 1.			Experiment 2.			Experiment 3.+		
No. of days after drying.	Count.	Survival in percent.	No. of days after drying.	Count.	Survival in percent.	No. of days after drying.	Count.	Survival in percent.
1	202×10^5		1	54×10^6		1	87×10^6	
21	110×10^5	54.4	17	40×10^6	74.0	7	107×10^6	100.0
						11	96×10^6	100.0
						15	80×10^6	91.6
						19	66×10^6	76.5
						4 months.	25×10^6	2.8%

+ The same preparation in a desiccator without vacuum in the refrigerator gave after 4 months a survival of 39.8% compared to 2.8% at room temperature.

TABLE 7.

T A B L E 7.

Survival of dried bacteria at room temperature under vacuum. (Bact. aerogenes A10C)

Experiment 1.			Experiment 2.			Experiment 3.		
No. of days after drying.	Count.	Survival in percent.	No. of days after drying.	Count.	Survival in percent.	No. of days after drying.	Count.	Survival in percent.
1	51×10^6		1	238×10^6		1	104×10^7	
28	10^3	< 0.1	14	758×10^5	3.2	6	512×10^6	4.9
			15	19×10^5	0.8	8	345×10^5	3.3
			16	42×10^5	0.5	19	403×10^4	0.4
			18	125×10^3	0.05	23	147×10^4	0.1
			23	90×10^3	< 0.05			

not be rubbed off and were then put between folds of sterile filter paper in a dish to keep the disks flat. The preparations were first kept under vacuum in a desiccator over anhydrous calcium chloride. Later on it was found that repeated removal and reapplication of vacuum when the preparations were taken for irradiation brought about a rapid destruction of the bacteria and therefore the dishes were kept in the desiccator at room temperature without vacuum.

The survival percentage at room temperature with and without vacuum are given in Tables 7 and 8. The survival was estimated by determining the viable count before and after drying. The cultures were suspended in sterile $\frac{1}{4}$ strength Ringer's solution and 1 ml. aliquots of suitable dilutions taken for counts. The medium used was "complete" agar medium without any glucose. The colonies were counted after incubation for 48 hours at 37°C.

The results in Tables 7 and 8 showed that survival under vacuum, with occassional periods at normal pressure, was below 1% after 19 days whereas without vacuum ~~after~~ it was 55 to 76% after 17 to 21 days.

Irradiation.

These dried preparations were then irradiated by putting the small disks covered with larger disks in the middle of a sterile petri dish (the same size as the window of the X-ray unit) one on top of another and

kept in position by a layer of cellophane and sterile cotton. The whole dish was covered by a piece of cellophane which had been sterilised along with the dish keeping it in position under the lid. The dish so prepared was held at the window of the apparatus and exposed to different dosages for different times. The survival rate of the organisms on irradiation was estimated by determining the viable count before and after irradiation. A dosage of 45,000 r gave a survival of 1.5%, 90,000 r gave 0.2 to 1% and 135,000 to 180,000 r gave a survival of 0.1 to 0.01%. This last survival rate was considered to be satisfactory for the production of an appreciable proportion of mutants. So a dosage of 135,000 to 180,000 r was chosen for the production of mutants.

The mutants were isolated by the method of Lederberg and Tatum (1946) which is based on the fact that from the plating of a mixed suspension of mutated and unmutated cells on a "minimal" agar medium only the latter will give origin, at first, to visible colonies; if, however, after a period of incubation a rich medium is added the mutants will then grow but will be distinguishable from the non-mutants because of their smaller colony size.

The irradiated cultures were reconstituted in $\frac{1}{4}$ strength Ringer's solution, immediately after the exposure, and suitable dilutions of the suspension

made so that 0.1 to 1 ml. would give a viable count of 50 to 200 cells. The "minimal" agar plates were prepared by pouring first a thin layer of the medium. Then a second layer, to which an aliquot of a suitable dilution of the irradiated culture had been added, was poured. After setting a third layer of the uninoculated medium was added. The plates were then incubated at 37°C. After 36 to 48 hours the colonies of the unmutated cells were large enough to be counted and their position marked. A fourth layer of "complete" agar medium was then added. After a further 24 hours incubation more colonies appeared and these were marked, picked to "complete" agar medium slopes, and tested for their ability to grow in "minimal" medium. Only a few of these were mutated colonies. In the later experiments, unirradiated preparations were examined for mutants at the same time as the irradiated ones following the same technique.

In the above technique, the first layer insured a uniform depth to all the colonies in the second layer and the third layer prevented growth from spreading. Spreading is more liable to occur at a glass or air interface than at the interface between two media. The third layer kept the cells in a colony from contaminating the entire plate in the next step of pouring the "complete" agar medium.

Both "minimal" and "complete" media had to be

modified because the colonies growing in the "minimal" agar layers produced gas by fermentation of glucose and split the surface obscuring the position of the colonies and giving spreading growth on addition of the "complete" medium. Glucose was therefore omitted from the "complete" agar medium. When the amount of glucose in the "minimal" medium was reduced from 2 to 0.5% gas production was still vigorous. Sucrose 0.5% also gave gas production though it was less vigorous than with glucose. With 0.1% glucose no gas was formed but the colonies became visible only after 5 days. With sodium citrate and sodium potassium tartarate as organic sources of carbon no gas was produced but appreciable growth took place only after 3 days. Finally, the amount of ammonium sulphate was increased from 1 g. to 5 g., glucose reduced to 0.5% and in this way a satisfactory medium with no gas production was obtained. The composition of the modified "minimal" agar medium was as follows:-

Glucose	5.0 g.
$(NH_4)_2SO_4$	5.0 g.
KH_2PO_4	3.46 g.
$MgSO_4$, $7H_2O$	0.038g.
Agar	15.0 g.
Distilled water.....	1000 ml.

pH adjusted to 7 with sodium hydroxide.

This modified "minimal" agar medium was used only

TABLE 9.

Viable counts of unirradiated and irradiated preparations by different methods.

Expt. No.	Irradiation dose. Roentgens.	Viable counts by method.				
		(1)	2		3	
				% of (1)		% of (1)
1.	0	83×10^6	74×10^6	88.6	231×10^5	27.8
		150×10^6	155×10^6	100.0	73×10^6	48.5
	180,000	302×10^1	184×10^1	60.9	34×10^1	11.1
		116×10^2	47×10^2	40.5	46×10^1	4.0
2.	0	38×10^6	43×10^6	100.0	68×10^5	18.1
		95×10^6	101×10^6	100.0	363×10^5	38.2
	180,000	94×10^2	64×10^2	67.6	21×10^2	22.6
		128×10^2	79×10^2	61.3	20×10^2	15.6
3.	0	48×10^6	43×10^6	90.5	76×10^5	15.9
		112×10^6	118×10^6	100.0	294×10^5	26.4

for this technique. When the term "minimal" medium is used in other contexts it refers to the original medium given on p. 24.

Although this screening method brings about a considerable saving in time, labour, and materials as against that of picking of individual colonies and testing of each in "minimal" medium, it was found that there was a great difference between the number of colonies growing in "minimal" medium as in this method and the number of colonies obtained in the viable count using "complete" agar medium without glucose. Therefore, viable counts of both unirradiated and irradiated preparations were made in three different ways, following the stratification method of Lederberg and Tatum but using different media. (1) Three layers of "complete" agar medium were used without glucose. (2) Three layers of "minimal" agar medium were used with a fourth layer of "complete" agar medium poured immediately after the third layer had set. (3) Three layers of "minimal" agar medium were used and after incubation for two days at 37°C a fourth layer of "complete" agar medium without glucose was added. The results obtained in three representative experiments are shown in Table 9.

From Table 9 it can be seen that with unirradiated preparations the viable counts by methods (1) and (2) did not differ. With irradiated preparations, the count by method (2) was from 40.5 - 67.6% of that by

method (1). Method (3) gave a much lower count than by the other two methods. This might be due to the fact that the "minimal" medium by itself did not provide optimum conditions for growth of colonies. With unirradiated preparations the count was from 15.9 - 48.5% of that by method (1) and with irradiated preparations from 4.0 to 22.6%.

The growth of irradiated bacteria in "minimal" medium by this stratification method was much less than that of the unirradiated bacteria even when a rich supplement was added immediately. Delay in the addition of the supplement affected the irradiated bacteria more than the unirradiated bacteria. Thus, it looks, as if the irradiated bacteria require immediate addition (compare method 2 with method 3) of "complete" medium in large amounts (compare method 2 with only a fourth layer of "complete" medium with method 1 where all layers are of "complete" medium.

These effects might be due to a lowered viability produced by irradiation, the irradiated cells dying rapidly unless the conditions are optimum regarding the nutrient medium, aeration, etc.

Apart from these effects, probably non-genetic in nature, there are numerous references in genetic literature to lowered viability associated with mutations, beginning with Muller's reference in 1918-1919 to the great majority lowering the viability in Drosophila.

TABLE 10.

Results of irradiation of cultures in the dry state.

Expt. No.	Dose in roentgens Time in Mins.	Viable count of dried preparations Before Irradiation. 10^5	Viable count of dried preparations After Irradiation. 10^2	Survival rate per 1000	No. of colonies developed "minimal" medium.	No. of colonies developed "minimal" medium. plus "complete" medium	No. of colonies developed "minimal" medium. plus "complete" medium	No. of colonies developed "minimal" medium. plus "complete" medium	No. of mutants.	No. of mutants.	% rate of mutation.	Mutant Nos.
					Unirrad.	Irrad.	Unirrad.	Irrad.	Unirrad.	Irrad.		
1.	135,000 (90)	67	105	1.5		100		6		1	1	3A3
2.	135,000 (90)		58	0.3		1220		27		5	0.4	4A3, 4A4, 4A31, 4A15, 4A10.
3.	135,000	350	5	0.15		122		2		1	0.8	5A2.
	180,000 (120)	350	24	0.07		272		8		1	0.35	5B8
	0	350			181		0		0			
	0	350			555		10		1			5C1
4.	0	505			872		6		0			
5.	135,000 (90)	40	87	2		1541		7		2	0.13	7A4, 7A2.
	180,000 (120)	40	11	0.3		735		15		4	0.6	$7B_3$, $7B_1$, $7B_2$. $7B_5$.
6.	180,000 (120)	1073	302	0.3		1468		17		6	0.4	$9B_1$, $9B_{17}$, $9B_2$, $9B_{16}$, $9B_4$, $9B_6$.
	0	1073			889		8		0			
7.	180,000 (120)	1165	116	0.1		198		10		0		
	0	1165			1809		4		0			
8.	180,000 (120)	995	91	0,1		1364		18		3	0.2	$11B_{16}$, $11B_1$, $11B_2$
	0	995			2097		9		0			
9.	180,000 (120)	663	56	0.1		1641		11		1	0.1	$12B_{19}$.
	0	663			1728		17		0			
				Total	8131	8661				24		

Although no definite data on this point were obtained with the mutants of Bact. aerogenes A10C clear evidence of this with a biotin-less mutant of Aspergillus nidulans and many other mutants has been obtained by Pontecorvo (unpublished). The biotin-less mutant survives only for a few hours in "minimal" medium unless the biotin is added. Thus, it is almost certain that with Lederberg and Tatum's technique certain mutants cannot survive on the "minimal" medium until "complete" medium is added and would consequently be missed. Therefore, though this method involves less labour and time than the picking of colonies and testing each one, the results cannot be used without qualifications in any quantitative expression of dose and survival, of dose and mutation rate, of proportions of "adaptable" and "non-adaptable" mutants or in speculations regarding the ease of mutation of particular genes.

The results of nine irradiation experiments involving the isolation of mutants are brought together in Table 10. From this table it can be seen that of 8131 colonies examined from unirradiated preparations only one was a mutant. Of 8661 colonies examined from irradiated preparations twenty-four were mutants. The mutation rate in the different experiments varied from 0.1 - 1%. The indication is that a number of factors should be carefully controlled to reduce this variation in different experiments.

These mutants were morphologically the same as

TABLE 11.

"Adaptable" and "non-adaptable" mutants.

Type	Mutant No.	Exp. (Table 9) from which the mutants are obtained.
"Adaptable"	$4A_{15}$, $4A_{10}$,	2
	$5B_8$,	3
	$7A_2$, $7A_4$, $7B_1$, $7B_2$, $7B_5$,	5
	$9B_2$, $9B_{16}$, $9B_4$, $9B_6$,	6
	$11B_2$,	8
"Non-adaptable"	297, 506, 518,	2+
	$3A_3$,	1
	$4A_3$, $4A_4$, $4A_{31}$,	2
	$5A_2$.	3
	$7B_3$.	5
	$9B_1$, $9B_{17}$,	6
	$11B_4$, $11B_{16}$,	8
	$12B_{19}$,	9

+This is from Experiment 2, Table 6.

the parent strain, that is, they were gram-negative, non-motile rods, and biochemically showed negative methyl red, positive Voges-Proskauer and negative indole reactions like the parent strain. They all produced acid and gas with glucose and lactose. They did not grow in Koser's citrate medium unlike the parent strain but this result could be expected as these strains would not grow in any synthetic medium without additional nutrilites.

Differentiation into "adaptable" and "non-adaptable" mutants.

The mutants encountered in this work have been observed to be of two types. While both types require for immediate growth in the "minimal" medium the addition of a particular nutrilite or mixture of nutrilites, one type will grow without this addition after a lag period of twenty-four hours or more. This type has been called "adaptable". A "non-adaptable" mutant will not grow without its particular nutrilite. (Ability to grow in "minimal" medium was tested usually for 3 days at 37°C though in a few cases it was checked for a longer period.)

In the present investigation, one notable feature of the results has been the high proportion of the mutants which are of the "adaptable" type. Thus of the 24 mutants listed in Table 9 thirteen are of the "adaptable" type. The two types of mutants are differentiated in Table 11.

TABLE 12.

Identification of the mutants.

Mutant No.	Nutrilite required.
Mutant from the unirradiated culture. $5C_1$ (non-adaptable)	Uracil.
X-ray-induced mutants Non-adaptable mutants 297, 506, (Obtained from the same batch.)	Histidine.
518	No growth with the single substances tested. Grew well with yeast extract.
$3A_3$	Nicotinic acid or amide.
$4A_3$	Cystine or methionine.
$4A_4$	Methionine.
$7B_3$	Serine or glycine.
$9B_1$	Threonine.
$12B_{19}$	Arginine.
$5A_2$	No growth with the single substances tested. Grew well with yeast extract.
$4A_{31}$	Adenine.
$11B_{16}$	Aneurine.
$9B_{17}$	Slight growth with leucine after two days. No growth with others.
$11B_1$	Died before it could be identified.
Adaptable mutants.	
$4A_{15}$	Aneurine.
$4A_10$	Uracil.
$7A_2$	Aneurine.
$7A_4$	Cystine or methionine.
$9B_2$	Histidine.
$11B_2$	Asparagine.
$^+7B_1$	Good growth with yeast extract. Fair growth with casein hydrolysate.
$^+7B_2$	Good growth with yeast extract. Very slight with casein hydrolysate and peptone.
$^+7B_5$	Good growth with yeast extract, fair with peptone and slight with casein hydrolysate.
$^+7B_8$	Good growth with yeast extract, slight growth with casein hydrolysate. No growth with peptone.
$^+9B_4$	Good growth with yeast extract, slight with casein hydrolysate. No growth with peptone.
$^+9B_{16}$	No growth with any simple substance tried. Good growth with yeast extract and very slight with casein hydrolysate and peptone.
$^+9B_6$	Good growth with yeast extract.

$^+$These adaptable mutants grew on the surface of "minimal" agar medium plates whether any added nutrilite was present or not. They did not grow well in broth with any of the simple substances. There was some slight growth in two days with some amino acids like histidine, serine, asparagine, glutamic acid and proline. A mixture of thymine and proline gave slight growth in two days in the case of $7B_1$ and $5B_8$. All of them gave good growth with yeast extract and variable growth with casein hydrolysate and peptone.

Further studies on the "adaptation" of these mutants are presented in Part II.

Identification of mutants.

The mutant strains were tested in a systematic manner to find what substance or substances had to be added to the "minimal" medium in order that growth should take place. The method followed was the same as that used for the identification of the spontaneous mutants of Bacterium prodigiosum (p. 25). Washed suspensions of the strains were spread on prepared plates of minimal agar medium and crystals of different substances put on different marked spots of the plate. A good confluent growth around any one of these spots of the plate indicated that the strain required the particular substance there for its growth. The mutant strains were then designated (following Beadle and other workers) by adding".....less" to the substance required for growth, e.g. histidine-less, aneurin-less, arginine-less, etc. In view of the very preliminary nature of the biochemical work done, these designations do not imply a specific block but only that the substance included in the description is by itself sufficient to promote growth when added to the "minimal" medium.

The mutants with the particular nutrilites required are given in Table 12.

TABLE 13.

Classification of the mutants according to nutrilite requirements.

Designation of the mutant.	No. of mutants.	Mutant No.	
		Non-adaptable.	Adaptable.
Nicotinic-less	2	893 and $3A_3$	
Histidine-less	3	297, 506	$9B_2$
Uracil-less	2	$5C_1$	$4A_{10}$
Cystine-less or methionine-less	3	$4A_3$ and $4A_4$	$7A_4$
Serine-less or glycine-less	1	$7B_3$	
Threonine-less	1	$9B_1$	
Arginine-less	1	$12B_{19}$	
Adenine-less	1	$4A_{31}$	
Aneurine-less	3	$11B_{16}$	$4A_{15}$ and $7A_4$
Asparagine-less	1		$11B_2$

Of the thirty mutants obtained, three spontaneous and twenty-seven induced, eighteen required simple substances as additions to the "minimal" medium, eleven did not grow with any simple substance but grew with yeast extract and one died before any test could be performed.

The eighteen mutants whose nutrilite requirements are known are classified in Table 13, according to their nutrilite requirements.

The mutant types were thus similar to those obtained with Neurospora, Aspergillus, Penicillium, Ophiostoma, E. coli, Acetobacter melanogenum and Bacillus subtilis. As ten out of thirty mutants are unidentified and also because of the small number of mutants obtained no conclusion can be made regarding the frequency of any particular type of mutation.

SECTION 3.

CHARACTERISATION OF THE MUTANTS.

The biochemical characteristics of all those mutants whose nutrilite requirements were known were studied in more detail in order to determine whether related compounds would meet their growth requirements or not and at what stage the block in the synthesis of the nutrilite occurred. Such studies would show whether these mutants were suitable for microbiological assays and also throw some light on the biosyntheses of vital cellular constituents,

the two objects with which this present investigation was undertaken. Because of the difficulty of obtaining some of the compounds required and the limited time available no comprehensive or exhaustive study could be undertaken. Therefore of the two methods of approach which were available, (1) a thorough investigation of one series of reactions, and (2) preliminary work on several series of reactions the latter method was chosen. This preliminary work should at least show (1) to what extent the paths of biosyntheses in Bact. aerogenes follow those in Neurospora, Penicillium, Ophiostoma, E. coli and other micro-organisms; and (2) perhaps supply some information on missing links in one or other of these reactions.

Experimental method:- The ability of different related substances to meet the growth requirements of each mutant was determined by the addition of a few crystals of the substance to "minimal" broth medium inoculated with the mutant strain under test. Growth, after incubation overnight at 37°C, was recorded by visual turbidity and was not measured quantitatively.

Nicotinic-less mutants Nos. 893 and 3Az.

The following substances were tested for their ability to replace nicotinic acid as an essential nutrilite for the growth of the mutants Nos. 893 and 3Az :- nicotinamide, picolinic acid, quinolinic acid,

TABLE 14.

Activity of certain compounds for the growth of the nicotinic-less mutants 893 and 3A3.

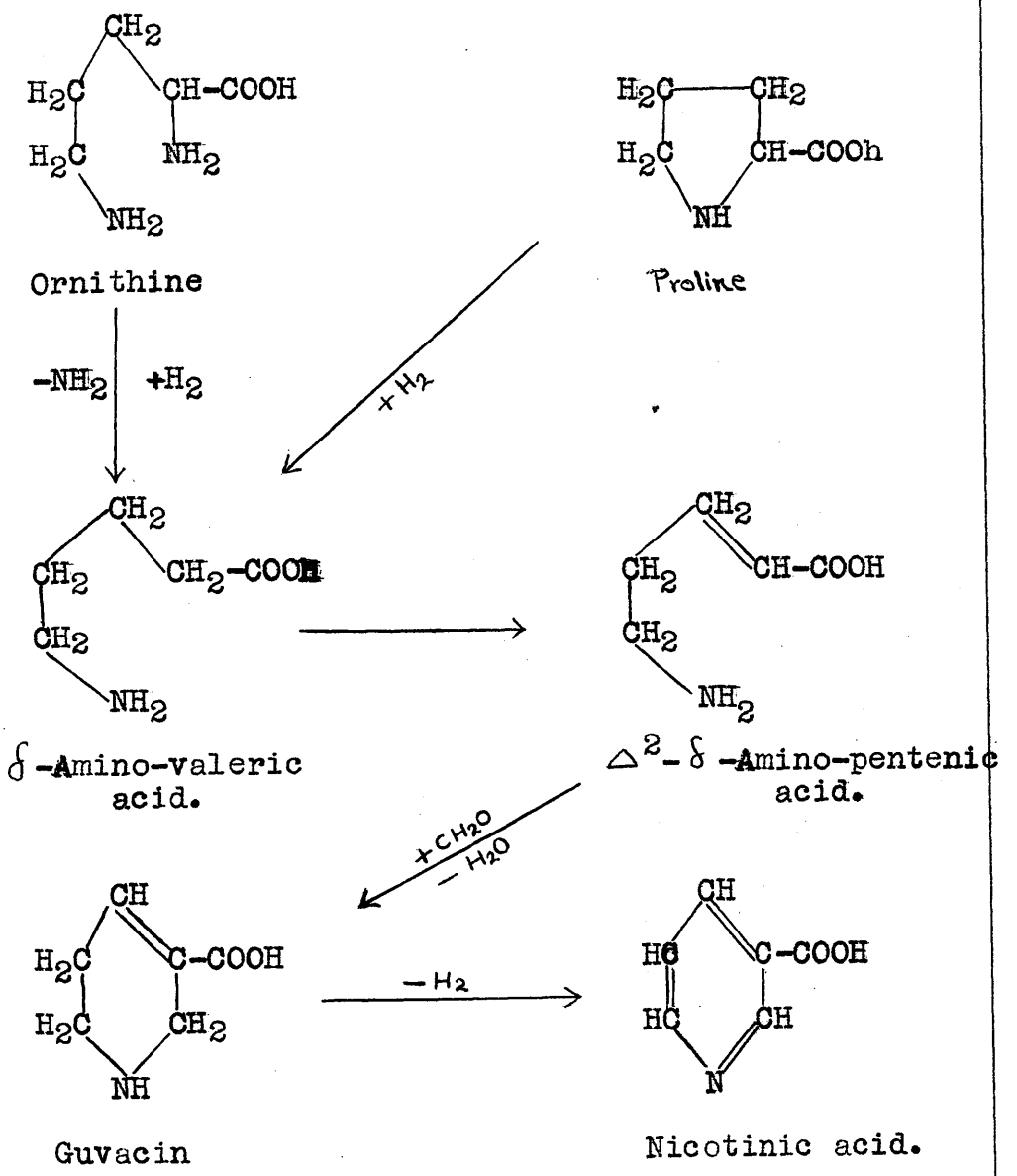
Compounds	Growth response. (24 hours, 37°C.) 893	3A3	Neurospora mutants.
Nicotinamide	+	+	+
Pyridine	-	-	-
Piperidine	-	-	-
Picolinic acid	-	-	not tested
Quinolinic acid	+	+	not tested
DL-ornithine	-	- (very slight growth in 3 days.)	-
L-proline	-	- (slight growth in 2 days.	-
∝-amino-n-valeric acid.	-	-	-
L-tryptophan	-	-	-
Trigonelline	-	-	-

L-amino-n-valeric acid, trigonelline, pyridine, piperidine, ornithine, proline and tryptophan.

Picolinic and quinolinic acids were included as α-mono- and di-carboxylic acid derivatives of pyridine. Trigonelline, pyridine and piperidine were included as being derivitavies of or related in basic structure to nicotinic acid.

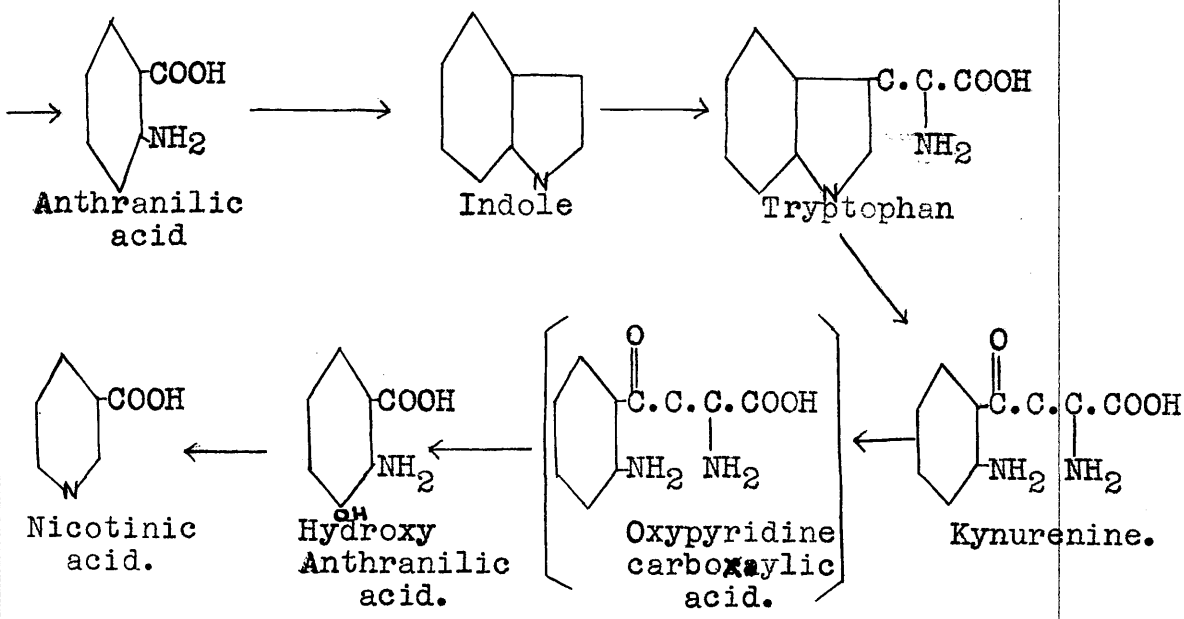
The results are shown in Table 14. For comparison, the effect of these substances on the growth of the nicotinic-less mutants of Neurospora (Bonner and Beadle, 1946) have been included in the Table. Of the substances tested only nicotin-amide and quinolinic acid could replace nicotinic acid. Quinolinic acid has also been shown to replace nicotinic acid for Proteus and dysentery bacilli (Dorfman, Koser, Reames, Swingle and Saunders, 1939).

According to the theory postulated by Guggenheim (1940) for the biogenesis of nicotinic acid the precursors include ornithine and proline, the synthesis being represented by the following scheme:-



Both ornithine and proline failed to replace nicotinic acid in the growth of mutants 893 and $3A_3$ although a slight response in 2 days with proline and a very slight response with ornithine in 3 days was given by mutant $3A_3$. These negative results may only mean that the block on the synthesis occurs at a later stage. The compounds required to test this have not

been available. On the other hand, it may be that, for these mutants, the synthesis follows another route such as that suggested by Beadle and his co-workers (Beadle and Bonner, 1946; Beadle, Mitchell and Nye, 1947; Nutrition Reviews, 1948; Mitchell and Nye, 1948; Bonner, 1948) for the Neurospora mutants, thus:-



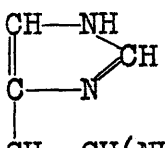
Addition of tryptophan to the "minimal" medium did not bring about growth of either No. 893 or 3A3. Tryptophane had no sparing effect on the amount of nicotinic acid or nicotinamide required by either of the strains.

The block may again be in the latter stages of the synthesis but owing to the non-availability of the necessary compounds it has not been possible to investigate further the mechanism of the biosynthesis

of nicotinic acid in the nicotinic-less mutants of Bact. aerogenes.

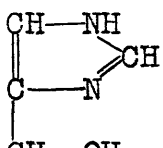
Histidine-less mutants Nos. 297, 506, and $9B_2$.

4-hydroxy-methyl glyoxaline hydrochloride and benziminazole, compounds structurally similar to histidine, were not able to replace the latter as essential nutrilites for these mutants nor were combinations of 4-hydroxy-methyl glyoxaline hydrochloride with alanine or glycine.



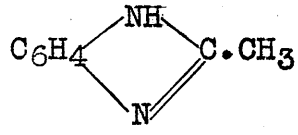
$CH \cdot CH(NH_2)\ COOH$

Histidine.



$CH_2\ OH$

4-hydroxy-methyl glyoxaline.



Benziminazole.

It was possible that one of the mutants might produce a precursor of histidine, one of the intermediates in the biosynthesis, which could be utilised by one of the other histidine-less mutants. For the investigation of this point, the three mutants were grown in the "minimal" broth medium with the minimum quantity of histidine for two days and the cultures boiled. Tubes of "minimal" broth medium were inoculated with each of the three mutants separately and those for each mutant divided into four parts - One part (1) was used as a control to which no boiled extract of any mutant was added. To a second part (2) boiled

extracts of the other two mutants were added. With all the three mutants, there was no growth in the controls (1) nor in (2) showing that the boiled extracts did not contain any histidine derivative or precursor available for the particular mutant. With 297 and 506, boiled extracts of each other or of $9B_2$ did not bring about any growth. This may mean that 297 and 506 were identical mutants with the block in the synthesis of histidine arising at the same place. This view was supported by the facts that the two mutants were obtained from the same irradiated batch and that both mutants gave identical dose-response curves for histidine (see Part III). With $9B_2$, growth was obtained on addition of boiled extracts of either 297 or 506. It seemed likely that 297 and 506 produced a histidine precursor which $9B_2$ could utilise instead of histidine. Therefore, the block in the synthesis of histidine by mutants 297 and 506 must have been at a stage after the synthesis of the intermediate which can be utilised for growth by $9B_2$. The block in $9B_2$ must have occurred at an earlier stage in the biosynthesis of histidine than in mutants 297 and 506. No further work has been done so far to isolate the histidine precursor for $9B_2$ produced by 297 and 506.

Adenine-less mutant $4A_{31}$.

This mutant $4A_{31}$ gave a reddish brown colour in

TABLE 15.

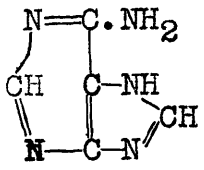
Activity of certain compounds for the adenine-less mutants.

Compounds	4A31.	Eight typical Neurospora mutants.
Adenine	+	+
Adenosine	+	+
Adenylic acid	+	+
Hypoxanthine	Not tested	+ (except one)
Xanthine	Slight	-
Guanine	+	-
Guanosine	+	-
Guanylic acid	+	Not tested
Yeast nucleic acid	Slight	Not tested
Adenine-cystosine di-nucleotide	Slight	Not tested
Cytidine	-	Not tested+
Uracil	-	-
Uric acid	-	-
Thymine	-	-
Histidine		-
Urea		-
Benzimidazole	-	Not tested
Allantoin	-	-
2,4,5,-triamino-6-hydroxy pyrimidine sulphate.	-	Not tested

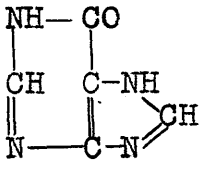
+ Cystosine was tested instead and did not promote any growth.

"complete" agar medium (compare with the adenine-less mutant 35203 of Neurospora and its genetic replicates (Mitchell and Houlahan, 1946.)). Some of the purines, pyrimidines, nucleotides and nucleosides were tested for their ability to replace adenine for this mutant. The compounds tested are shown in Table 15 together with results obtained for forty-five adenine-less mutants of Neurospora (Mitchell and Houlahan, 1946).

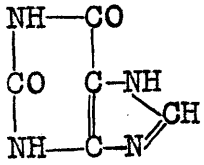
This mutant grew rapidly with adenine (6-amino-purine) and its derivatives, adenosine and adenylic acid. Unfortunately, hypoxanthine (6-oxy-purine) was not available for testing but xanthine (2, 6-dioxy-purine) allowed some growth to occur. Unlike the Neurospora mutants and also Ophiostoma mutants (Fries, 1946 a) and the mutant $4A_{31}$ grew fairly well in two days with guanine (2-amino-6-oxy purine) and its derivates guanosine and guanylic acid. Burkholder and Giles (1947) have obtained a similar mutant of Bacillus subtilis growing both with adenine and guanine. These results indicate that the block in the synthesis does not occur at the stage of amination of the purine ring but at some other stage. The formulas of these main compounds are given below.



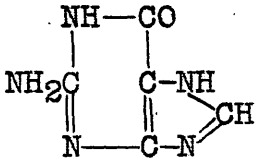
Adenine or 6-amino purine.



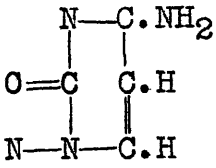
Hypoxanthine or 6-oxy purine.



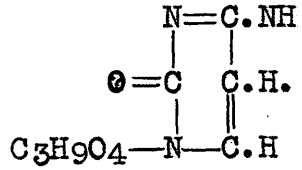
Xanthine or 2, 6-dioxy purine.



Guanine or 2-amino-6-oxy purine.



Cytosine or 3-amino-pyrimidine



Cytidine.

The more complex compounds containing adenine — adenine-cytosine-dinocleotide and yeast nucleic acid, promoted growth to a slight extent only, perhaps due to an inability to break down these products rapidly enough to make the adenine available. As with the Neurospora mutants, the pyrimidine compounds failed to allow growth.

Aneurin-less mutants, Nos. $11B_{16}$, $4A_{15}$ and $7A_2$.

These mutants have been tested for growth in the "minimal" medium supplemented with the breakdown products of aneurin, 4-methyl-5-β-hydroxyethyl thiazole

TABLE 16.

Growth of $4A_3$, $4A_4$, and $7A_4$, with various sulphur containing compounds.

Compounds.	Strains.		
	$4A_3$.	$4A_4$.	$7A_4$.
dl-methionine	+	+	+
l-cystine	+	-	+
Cystine hydrochloride	+	-	+
Thioformamide, SH-CHO	+	-	+
Thiourea	+	-	+
Phenyl thiourea	slight	-	slight
Thioglycollic acid	+	-	+
Thiodiglycol	slight	-	slight
1-amino-2-Napthol-4-sulphonic acid	slight	-	slight
Sodium-b-Napthoquinone sulphonate	slight	-	slight
Sulphanilic acid	-	-	-
Taurine	-	-	-
l-leucine	+	slight	+
Leucine (synthetic, Kahlbaum.)	-	-	-
Choline	-	-	-
Cystine and choline	-	-	-
Sodium sulphate Na_2SO_4	-	-	-
Sodium bisulphate $NaHSo_4$.	-	-	-
Potassium persulphate	-	-	-
Ammonium thiocyanate	-	-	-
Sodium sulphite Na_2SO_3	+	-	-
Sodium metabisulphite	+	-	+
Sodium hyposulphite $Na_2S_2O_4$	+	-	+
Sodium thiosulphate $Na_2S_2O_3$	+	-	+

and 2-methyl-4-amino-5-ethoxy - methyl pyrimidine, and with thioformamide one of the presumed intermediates in the synthesis of thiazole.

The "non-adaptable" mutant $11B_{16}$ grew with either aneurin or thiazole but not with the pyrimidine compound or the thioformamide. This indicated that with this mutant the block in the biosynthesis was in that of the thiazole moiety. The adaptable mutants $4A_{15}$ and $7A_2$ curiously enough grew with all the compounds.

Cystine-less or methionine-less mutants, 4A3, 4A4, and 7A4.

These three mutants were unable to utilise sulphate for their complete sulphur requirements. They required more reduced forms of sulphur. Such strains should be called parathiotrophic in contrast to those utilising sulphate, euthiotrophic (Lwoff, 1932, quoted by Lampen, Roepke and Jones, 1946); Fries, 1946b). All of them utilised methionine and cystine except $4A_4$ which utilised only methionine. Other inorganic and organic sulphur compounds containing 6-, 4-, and 2- valent sulphur have been tested for their growth promoting activity for these strains. The result with these compounds are shown in Table 16.

It can be seen from the table that mutants $4A_3$ and $7A_4$ differed only in their growth response to sodium sulphite. $4A_4$ was more stringent in its requirements and responded only to methionine containing

a 2- valent sulphur. Choline as a source of methyl group did not give growth in the presence of cystine. It is quite possible that homocystine might have promoted growth but this substance was not available. All strains grew with L-leucine but failure to grow with synthetic leucine (Kahlbaum) showed that this might be due to the presence of some impurity. It should be pointed out that L-leucine is usually considered to contain some nor-leucine which is very similar to methionine in structure.

$CH_3.S.CH_2.CH_2.CH(NH_2).COOH.$

Methionine or α-amino-γ-methylthiol-n-butyric acid.

$CH_3.CH_2.CH_2.CH_2.CH(NH_2).COOH.$

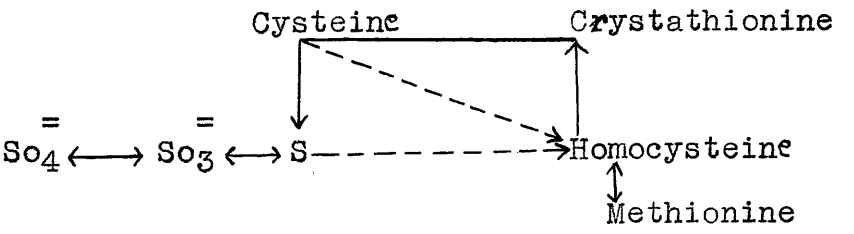
Nor-leucine or α-amino-n-caproic acid.

$4A_3$, and $7A_4$ utilised cystine, cysteine and other bi-valent forms of sulphur in simple organic compounds like thioformamide, thiourea and thioglycollic acid. They utilised the sulphur of the more complex compounds to a slight extent or not at all. No. $4A_3$ utilised 4-valent sulphur e.g. sulphite while $7A_4$ was unable to do so. Both the mutants utilised still more reduced forms of sulphur present in sodium metabisulphite, sodium hydrosulphite and sodium thiosulphate. They were unable to utilise sodium sulphate, sodium bisulphate and potassium persulphate.

Thus these strains can be classified into the following types. (1) Those which grew with methionine

only. This type seems unable to convert cystine to methionine. From the present work it was not possible to indicate at what stage this block in the conversion took place. (2) Those which grew with cystine, cysteine and methionine. This group can be divided into other two types, one of which utilised $Na_2S_2O_4$, $Na_2S_2O_3$, thioglycollic acid etc. and sodium sulphite while the other utilised $Na_2S_2O_4$, $Na_2S_2O_3$, thioglycollic acid etc. but not sodium sulphite. Similar types have been obtained in parathiotrophic forms of E. coli (Lampen, J.R., Roepke, R.R. and Jones, M.S., 1947) and of Ophiostoma multiannulatum (Fries, 1946b)

The behaviour of these three types with different sulphur compounds gives some support to the scheme put forward by Lampen, Roepke, and Jones (1947) for the possible interconversion of sulphur compounds in E. coli.



$\overset{=}{So_4}$ is first reduced to $\overset{=}{So_3}$, then to sulphide and forms cysteine and homocysteine and methionine.

Mutant $4A_3$ has lost the ability to reduce sulphate to sulphite whereas mutant $7A_4$ has lost the ability to bring about further reduction of sulphite. Mutant $4A_4$ could convert cysteine to methionine, either at

the cysteine to homocysteine stage or at the homocysteine to methionine stage.

Serine-less or glycine-less mutant, $7B_3$.

Either serine or glycine were able to satisfy the growth requirements of this mutant. Such mutants with alternative requirements for glycine or serine have been obtained from Acetobacter (Gray and Tatum, 1944), from E. coli (Horowitz, Houlahan, Hungate and Wright. (1946), and from Neurospora (Hungate, 1946). This biochemical relationship of serine and glycine was also in accord with the conversion of serine to glycine demonstrated in mammalian tissues. (Shemin, 1946).

$NH_2.CH_2.COOH$	$HOCH_2.CH(NH_2).COOH$
Glycine.	Serine or α-amino-β-hydroxy propionic acid.

Other substances like alanine, β-alanine, pyruvic acid, propionic acid and acetate were found to be incapable of supplying the essential growth requirements.

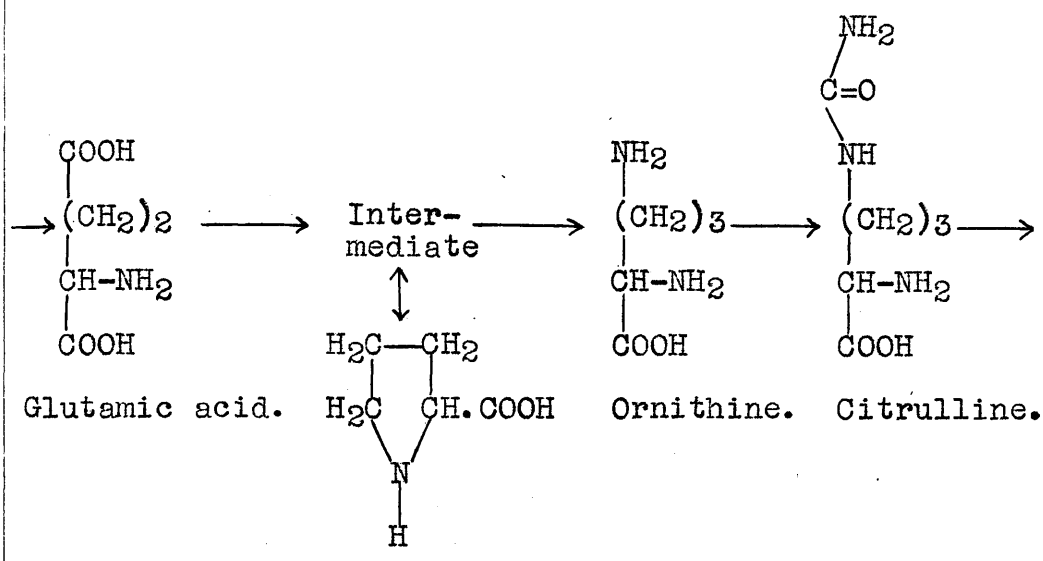
Threonine-less mutant, $9B_1$

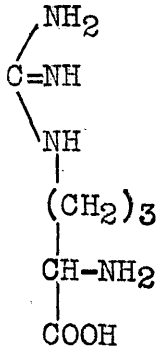
This mutant requiring threonine, α-amino-β-hydroxy-n-butyric acid, has been tested in addition to α-amino-butyric acid and sodium butyrate. These compounds were found to have no growth-promoting activity.

Arginine-less mutant, $12B_{19}$

The suggested scheme of biosynthesis of arginine α-amino -δ-guandine -n-valeric acid, in Neurospora (Srb, 1946; Srb and Horowitz, 1944), in Penicillium

(Bonner, 1946b) and in E. coli (Roepke, 1946) is:-





Arginine.

The following compounds, glutamic acid, proline, and ornithine present in the sequence of this reaction were tested for their growth activity and only ornithine was found to be active. Therefore it can be assumed that so far as this mutant is concerned the block in the biosynthesis occurred before the production of ornithine. Other substances like guanidine, α-amino-valeric acid, a mixture of α-amino-valeric acid and

guanidine, ammonium carbamate, urea, and creatinine did not have any growth-promoting activity.

SUMMARY.

Of the two different organisms examined for spontaneous and induced variations, Bact. prodigiosum had a high proportion of spontaneous mutants in its population and consequently further work on it had to be abandoned. Bact. aerogenes A10C gave only 2 mutant colonies out of 1935 colonies examined. This was considered to be a sufficiently low mutation rate and further work was conducted only on this organism.

Mutations were induced by irradiation with X-rays. Under the conditions employed irradiation of broth cultures was not successful. Therefore, further irradiations were conducted by irradiation of bacteria in the dry state. The fact that mutations could be induced in dried bacteria has not previously been reported.

Various methods of suspension of bacteria on different support were attempted. Finally measured amounts of a thick bacterial suspension were placed on thin permeable cellophane disks and these preparations dried under vacuum.

The survival rate after irradiation with 180,000 roentgen units was 0.1% to 0.03% and the mutation rate

varied from 0.1-1%.

After irradiation of bacteria in the dry state, the mutants were isolated by the method of Lederberg and Tatum. A critical examination of this method was conducted.

Thirty mutants were obtained of which seventeen were of the "non-adaptable" and thirteen the "adaptable" type. Nineteen of these "non-adaptable" and "adaptable" mutants were identified with regard to the particular nutrilite required to allow growth in "minimal" medium. The rest, most of them "adaptable", grew in the presence of yeast extract in "minimal" medium but were not further characterised.

Further biochemical studies were made on the mutants requiring nicotinic acid, histidine, aneurin, adenine, methionine, serine, arginine and threonine.

The growth requirements of the two nicotinic-less mutants were satisfied by nicotinic acid, nicotinamide or quinolinic acid.

The three methionine-less mutants were of three different types - one which grew only with methionine, another which grew with a 4-valent sulphur compound like sodium sulphite and a third which grew only with 2-valent sulphur compounds.

There were two kinds of strains requiring aneurin - the "non-adaptable" mutant grew with the thiazole moiety of aneurin and the two "adaptable" strains grew

with both moieties of the vitamin.

The serine-less mutant grew equally well with glycine thus demonstrating the inter-relations between these two amino-acids in bacterial metabolism.

The arginine-less mutant responded equally well to ornithine.

Of the three histidine-less mutants, the "non-adaptables", 506 and 297, seemed to be the same and the "adaptable" $9B_2$ different from the other two. The strain $9B_2$ grew on addition of boiled extracts of 506 and 297 to the "minimal" medium.

The adenine-less mutant grew rapidly with adenine, adenosine and adenylic acid and fairly well in two days with guanine, guanosine and guanylic acid. Xanthine allowed some growth to occur.

PART II.

STUDIES ON ADAPTATIONS.

INTRODUCTION.

When the X-ray induced mutants of Bact. aerogenes $A10_C$ were isolated it was found that they fell into two categories. In one category the mutants grew in "minimal" medium after a delay of 24 hours or so and in the other they failed to grow at all (maximum period of test was three days.) As stated in Part I such mutants have been designated "adaptable" and "non-adaptable". In those mutants which showed delayed growth in the "minimal" medium a gradual adjustment to a deficiency in the enviroment occurred which could justifiably be called adaptation and which enabled the mutant to dispense with a nutrilite which, at first, was essential.

Mutants which showed such adaptation in deficient medium have also been observed among mutants of Neurospora and Escherichia coli, Yeast and Ophiostoma. For instance adaptation was noted and exhaustively studied by Ryan and Lederberg (1946) and by Ryan (1946) in the leucine-less Neurospora mutant, 33757, obtained by ultra-violet radiation. Adaptation has also been reported in other Neurospora mutants such as

the mutant 1663 which required p-amino-benzoic acid (Tatum and Beadle, 1942b; Wyss, Lilly and Leonian, 1944), the isoleucine-valine mutant 16117 (Bonner, Tatum and Beadle, 1943) the proline-less mutant 21863 and the aneurine-less mutant 9185(Ryan, 1946). It was observed by Roepke, Libby and Small (1944) that nutritional mutants of E. coli such as the lysine-less mutant 152-171 and the histidine-less mutant 148-334 eventually grew normally in minimal medium. The double mutant of E. coli, requiring both threonine and leucine, became threonine - or leucine - independent depending on the media for growth.

In this work, however, the high proportion of adaptable mutants obtained, thirteen out of thirty seemed to be remarkable. Of these only six have been identified as requiring a simple nutrient factor. The others failed to respond to the simple nutrilites so far tested and required complex substances such as yeast extract. The "adaptation" of two of the mutants $4A_{15}$ and $4A_{10}$ was reversibly lost on passage through "complete" medium. These "adaptable" mutants can be of great interest in genetics and biochemistry. In some cases, the "adaptation" may result from back-mutation and selection, as shown for example by the leucine-less Neurospora mutant, 33757, analysed by Ryan (1946). But this is not the only possibility. The "adaptability" may be due to (1) an inheritable change causing the production of a changed form of the

enzymic, (2) an inheritable change entailing failure to produce an enzymic and opening the way for an alternative enzymic system to be brought into operation (Hinshelwood, 1946): or (3) the inactivation of a gene which, in the normal strain, controls directly or indirectly the rate of growth of a plasmagene identical with, or the precursor of an enzyme. (Spielgelman, 1946).

By comparing *in vitro* suitable enzymic systems of the parent strain with those of "adaptable" mutants it may be possible to investigate the first and second alternatives. The third alternative can be verified by investigating whether continued subculture of an "adaptable" mutant on a medium supplying the growth factor ultimately leads to the irretrievable loss of the "adaptability", that is, to the loss of every particle of the plasmagene. (Sonneborn, 1947). Some indication of the latter possibility was shown by the fact that the "adaptable" nicotinic-less mutant $3A_3$ on repeated subculture (12 passages) on "complete" medium became irreversibly "non-adaptable." This loss of "adaptability" did not occur with mutant $4A_{15}$ after subculture for 12 passages on "complete" medium.

The "adaptability" was inheritable as shown for $4A_{15}$ and $4A_{10}$. Once these mutants attained maximum growth in "minimal" medium they continued to grow without any delay on successive transfers to the same medium.

The main question which arises in connection with these "adaptable" mutants is whether or not the "adaptation" occurs by mutation and selection in any one particular case. This point has been investigated with eight "adaptable" mutants first by ascertaining whether they contain both nutrient - dependent and - independent cells and secondly by ascertaining whether or not adaptation occurs without cell multiplication. The studies made are entirely of a preliminary nature but may be of interest so far as they go.

It should be emphasised here that the required time for adaptation, delay in growth, depends in the case of many mutants on the size of the inoculum, e.g. the mutants $7A_4$, $7A_2$, $4A_{15}$, $4A_{10}$ and $9B_2$ had different "lag" periods depending on the size of the inoculum. With larger inocula some growth took place within 24 hours but this was never as great as the growth with addition of the particular nutrilite required.

This difference in "lag" period depending on the size of the inoculum might be due to the fact that the larger inoculum supplies enough of the nutrilite required by autolysis or it might be that if "adaptation" occurs by mutation and selection with a large inoculum the chance of nutrient-independent cells being present from the start is greater. Ryan (1946) showed, similarly,

that uracil-dependent Clostridium septicum grew equally well with or without uracil when a large inoculum was used and variably with smaller inoculum.

Variations in the "adaptable" mutant strains.

Variations occurring in the "adaptable" strains were investigated in the following experiments. They might be entirely composed of "adaptable" cells or partly of nutrient-dependent and partly of mutrient-independent cells. The strains $7A_4$, $9B_2$, $4A_{10}$, $7B_1$, $7B_2$, $7B_5$, $9B_{16}$, $9B_4$ and $5B_8$ were examined by picking individual colonies and testing the ability of each to grow in "minimal" medium.

The method employed was as follows:- From a 24 hour "complete" medium broth culture 0.05 ml. of a suitable dilution which would give a few well-isolated colonies was spread over the surface of a prepared plate of "complete" agar medium and incubated overnight at 37° C. Thirty colonies were picked to slopes of "complete" agar medium. After 24 hours at 37° C. the growth from each slope was inoculated into "minimal" medium broth. The inoculum was standardised by suspending a small amount of the culture in 9 ml. of sterile ¼ strength Ringer's solution, the turbidity being adjusted so as to be just visible against printed matter. Suspensions of all the cultures were thus prepared, one standard loopful from each inoculated into 10 ml. of "minimal" medium broth and incubated at 37° C. Growth was determined by visual turbidity after 19 hours.

TABLE 18.

Variations in the different types of the histidine-less $9B_2$ and cystine-less $7A_4$.

Mutant.	No. of colonies picked.	Growth in "minimal" medium 37°C.		
		1 day.	2 days.	3 days.
$9B_2$ (7)	30	30-	17-, $1^{\pm}$, 4+, 8+++	2-, 3^{-}_{+}, $1^{\pm}$, 9+, 15+++
$9B_2$ (21)	30	30-	24-, 1+, 5++	1-, 1^{-}_{+}, 11+, 17+++
$9B_2$ (11)	30	1^{-}_{+}, 29-	16-, $5^{\pm}$, 1+, 8+++	6-, $4^{\pm}$, 6+, 14+++
$7A_4$ (30)	30	$25^{\pm}$, 5+	30+++	
$7A_4$ (23)	30	3-, $7^{\pm}$, 11+, 9++	2-, 1++, 27+++	2-, 28+++
$7A_4$ (15)	30	7^{-}_{+}, $22^{\pm}$to+, 1+++	30+++	
$4A_{10}$(20)	30	30-	17-, 13+	16-, 1+, 13++

The symbols indicate:- - no growth, $^{-}_{+}$ trace, $^{\pm}$ very slight, + slight, ++ moderate, and +++ good growth and the figures the number of colonies giving such growth response.

TABLE II.

Variation in the "adaptable" mutant strains of Bact. aerogenes A10C.

Mutant.	No. of colonies picked.	Growth in "minimal" medium 37°C.		
		1 day.	2 days.	3 days.
$7A_4$	30	2-,4±,24++	1-,29+++	1-
$9B_2$	30	19-,6++,5+++	15-,10++to+++, 5+++	12-,3++,15+++
$4A_{10}$	27	19-,11++	3-,12±,15+++	2-,2±,11++, 15+++
$7B_1$	31	6-,2++,23+++	5-,26+++	4-,27+++
$7B_2$	25	3-,22+++	2-,23+++	2-,23+++
$7B_5$	30	2-,1±,27+++	1-,29+++	1-,29+++
$9B_{16}$	30	7-,10±,13+++	7-,23+++	7-,23+++
$9B_4$	30	3-,1±,26+++	1-,29+++	1-,29+++
$5B_8$	30	6-,1±,23+++	4-,26+++	4-,26+++
$7A_2$	30	30-	30+++	
$4A_{15}$	30	26-,4+++	25-,5+++	17-,3±,1+, 4++,5+++

The symbols indicate:- - no growth, ± very slight, + slight, ++ moderate, and +++ good growth, and the figures the number of colonies giving such growth response.

The results are given in Table 17.

The results show that with the exception of $7A_2$ there were three or four different types in each strain. Type (1) grew well within one day; (2) grew slightly in one day and moderately or well in two days; (3) grew well in three days; (4) did not grow in three days. The proportions of each type present varied with each strain. The histidine-less mutant $9B_2$ and and uracil-less mutant $4A_{10}$ gave all types, $9B_2$ showing the greatest number of Type 4.

Growth of three different types of cystine-less mutants $7A_4$ was re-examined for variation as before by plating, picking colonies to "complete" medium and then testing for rate of growth in "minimal" medium. $7A_4$ (30) had grown well in one day i.e. was entirely cystine-independent (Type 1) $7A_4$ (15) (Type 2) had shown slight growth in one day and good growth in two days and $7A_4$ (23) (Type 4) had failed to grow in 3 days in "minimal" medium and was entirely cystine-independent. On re-examination of 30 colonies (Table 18), $7A_4$ (30) had all colonies showing slight to very slight growth in one day and good growth in two days. Colonies from $7A_4$ (15) gave one whish was entirely independent of cystine while the remainder were of Type 2, some giving only straes in one day. The cystine-dependent type $7A_4$ (23) however gave colonies of varying response only two of which were entirely cystine-dependent.

From histidine-less mutant $9B_2$ strains of all three types, i.e. $9B_2$ (7) (Type 1,) $9B_2$ (21) (Type 2) and $9B_2$ (11) (Type 4) on re-examination yielded colonies of all types irrespective of the type first isolated. Similar results were obtained with a Type 4 strain of the uracil-less mutant $4A_{10}$ (20).

Ryan (1946) found the same phenomenon of nutrient-dependent and -independent types arising from either leucine-dependent or -independent strains of Neurospora, from uracil-dependent or -independent strains of Clostridium septicum and histidine-dependent or -independent strains of E.coli. (Ryan, 1946; Ryan, Schneider and Ballentine, 1946). Ryan concluded that adaptation was due to back-mutation and selection.

The results presented here appear to support Ryan's conclusions. They are, however, open to criticism since the cultures had been maintained on "complete" medium for about 5 days before re-examination and had thus a chance to reach equilibrium following mutation and selection if these were involved. The apparent instability of the strains could only be satisfactorily confirmed or disproved by the examination of colonies from single colony isolates at different times after their isolation. If mutation and selection are involved, the nutrient-dependent types should give an increasing proportion of nutrient-independent colonies, while the nutrient-independent types should give an

increasing proportion of nutrient-dependent colonies, until within five days or less both would be the same.

ADAPTATION WITHOUT CELL MULTIPLICATION.

Stephenson and Stickland (1932, 1933) and Yudkin (1932) showed that a washed suspension of coli could adapt itself to growth on formate, with the production of the enzyme formic hydrogenylase, without cell multiplication. This could occur only in nutrient broth favouring maximum growth but before any cell multiplication took place. Stephenson and Yudkin (1936) showed also that galactose adaptation in Saccharomyces cerevisiae could occur slowly in phosphate buffer solution without any nitrogen source and without any significant cell division, confirming the work of Dienert (1900) and others who had shown that it was possible for washed yeast suspensions in phosphate buffer containing galactose to become adapted. Spiegelman and collaborators (1946) in their detailed investigations on galactose adaptation in yeast confirmed that adaptation could occur in the presence of phosphate buffer and substrate. Hegarty (1939) showed that galactose adaptation occured in washed suspensions of Streptococcus lactis in the absence of either nitrogen source or cell division. That adaptation could occur in the presence of phosphate buffer and the substrate without cell division was also

shown by Pollock, Knox and Gell (1942). Pollock and Knox (1943), Knox and Pollock (1944) and Pollock (1945, 1946) with "tetrathionase" of Bact. paratyphosum B. and "nitratase" of Bact. coli, (Intermediate, Type 1, No. 1443) isolated by Pollock (1946). It therefore appeared to be of interest to determine whether "adaptation" of the "adaptable" mutants isolated in the present investigation could take place under similar conditions.

A thick bacterial suspension of the "adaptable" mutant was introduced into three different kinds of "adapting" medium incubated at 37°C and the occurrence of "adaptation" investigated by the inoculation of aliquots of this suspension at different intervals into "minimal" medium. It could be reasonably assumed that if adaptation occured the delay in growth in "minimal" medium would be reduced and more growth would take place in the same interval of time. Consequently if an increase in the amount of growth produced in "minimal" medium occurred with increasing time of contact with the "adapting" medium, this could be taken as evidence of adaptation.

Mutants:- The mutants used in most experiments were two strains of the "adaptable" histidine-less mutant $9B_2$ obtained when variation in $9B_2$ was being investigated. The two strains were $9B_2$ (7) which grew to some extent in "minimal" medium within one day but gave more growth in the presence of histidine and $9B_2$ (21) which grew

only after two days (using a small inoculum). In one experiment, a strain of the cystine-less mutant $7A_4$ (23) was used.

Preparation of bacterial suspensions. Growth from 24 hour cultures on "complete" agar medium was suspended in $\frac{1}{4}$ strength Ringer's solution, centrifuged, washed twice, and the third suspension in Ringer's solution used after centrifuging for a minute at a low speed to bring down any clumps. The viable counts of these suspensions were of the order of 10^9 to 10^{11} bacterial cells per ml. The suspensions were diluted 1:3 in the final adapting medium.

The opacity and viable counts of the inoculum:- The opacity of the inoculum was measured on the Spekker absorptiometer. The viable counts were determined by serial dilution of the inoculum and plating 1 ml. aliquots in duplicates on "complete" medium without glucose.

Preparation of adapting media:- Three adapting media were used, (1) a mixture of phosphate buffer and ammonium sulphate with or without magnesium sulphate, (2) a mixture of phosphate buffer, ammonium sulphate and glucose, with or without magnesium sulphate, (3) "minimal" medium only. Ammonium sulphate was used here partly as the substrate for the production of adaptation. 1 part of Sorensen's M/15 phosphate buffer pH 7.0, 1 part of ammonium sulphate and other ingredients of

the adapting medium were mixed, the temperature raised to 37°C. and 1 part of bacterial suspension (also at 37°C.) added. The final concentration of ammonium sulphate was always maintained at 0.1%. Glucose was used in concentrations of 1% and 0.1% and magnesium sulphate in a concentration of 0.004%. The "minimal" medium used was that given on p. 24. The adapting media were sterilised by autoclaving at a pressure of 10 lb. for 10 minutes.

Method of investigating adaptation:- 1 ml. aliquots were pipetted from the mixture at intervals into 9 ml. of ¼ strength Ringer's solution and 0.05 ml. or 0.1 ml. of this 1:10 dilution or 1:100 dilution inoculated into tubes (usually six) of 10 ml. sterile "minimal" medium. The tubes, after shaking well, were incubated in a water bath at 37°C and the growth measured turbidimetrically with the Spekker absorptiometer.

Results.

(1) Adaptation in a medium containing phosphate buffer and ammonium sulphate, with or without magnesium sulphate.

The adaptation of mutants $9B_2$ (7), $9B_2$ (21) and $7A_4$ (23) was investigated in a medium containing 5 ml. of N/15 phosphate buffer, pH 7.0, 5 ml. of 0.3% ammonium sulphate and 5 ml. of bacterial suspension. When magnesium sulphate was used the amount added was 0.012%. The final concentrations were ammonium sulphate 0.1%,

TABLE 20.

Adaptation in a mixture of phosphate buffer, ammonium sulphate and magnesium sulphate.

Organism.	Exp. No.	Time in adapting media. Hours.	Inoculum.		Growth spekker reading.	
			Spekker reading.	Viable count. x 10^6.	24 hours.	48 hours.
$9B_2(21)$	1	0	0.101	178	0.315	0.636
		6	0.086	152	-	0.587
		19.5	0.092	162	0.300	0.601
		48.5	0.098	138	0.290	0.607
	2	0	0.844	1780	0.360	0.685
		12.5	0.820	1620	0.376	0.766
		48.5	0.770	1380	0.383	0.682
$9B_2(7)$	1	0	0.150	215	0.206	0.389
		4	0.146	214	0.212	0.431
		7	6.135	214	0.208	0.300
		19.5	0.140	227	0.219	0.435
		28	0.112	237	0.216	0.440
		49	0.112	199	0.221	0.446

TABLE 19.

Adaptation in a mixture of phosphate buffer and ammonium sulphate.

Organism.	Exp.	Time in adapting medium. Hours.	Inoculum.		Growth. Spekker readings.		
			Spekker reading	Viable count x10^6	24 hours.	43 hours.	63 hours.
$9B_2$(21)	1.	0		26	0.220	0.529	
		4		26	0.293	0.538	
		19		26	0.253	0.573	
		43		19	0.255	0.567	
		168		8	0.177	0.449	
	2.	0		70	0.146	0.295	
		19		65	0.202	0.336	
		43		47	0.181	0.333	
		72		34	0.178	0.315	
	3.	0		700	0.176	0.321	
		19		650	0.143	0.350	
		43		468	0.153	0.390	
		72		338	0.144	0.480	
$9B_2$(7)	1.	0	0.145	112		0.306	0.599
		2	0.151	93		0.276	0.560
		4	0.141	100		0.300	0.668
		7	0.139	98		0.296	0.664
		19	0.140	102		0.308	0.668
$7A_4$(23)	1.	0	0.112	79	0.235	0.498	
		2	0.100	69	0.259	0.579	
		4	0.109	65	0.262	0.503	
		7	0.107	68	0.259	0.579	
		19	0.108	67	0.265	0.583	
	2.	0	0.136	136	0.284	0.530	
		19	0.128	104	0.280	0.661	
		43	0.108	69	0.277	0.562	
		70	0.122	53	0.297	0.581	

glucose 1% and magnesium sulphate 0.004%. From this mixture 1 ml. was removed at intervals to "minimal" medium and the growth determined. The results obtained using this adapting medium, with and without magnesium sulphate are given in Tables 19 and 20.

It can be seen from Tables 19 and 20 that within 24 hours no increase took place in rate of growth with time of exposure in the "adapting" media. Longer incubation in the "minimal" media did not show any evidence of "adaptation" with the possible exception of Experiment 3, Table 19. The viable counts showed that no cell multiplication had taken place in the "adapting" media.

(2) Adaptation in a medium containing phosphate buffer, ammonium sulphate and glucose, with or without magnesium sulphate.

Adaptation of $9B_2$ (21) and $9B_2$ (7) was investigated in another medium consisting of 5 ml. of N/15 phosphate buffer 7.0, 5 ml. of a solution containing 0.3% ammonium sulphate and 3% glucose and 5 ml. of bacterial suspension. The final concentrations were glucose 1% and ammonium sulphate 0.1%. This medium was tested in order to see whether adaptation would take place in a medium approximating in composition to the "minimal" medium. It was thought that even if growth might eventually take place adaptation could occur before appreciable cell division. The results are recorded in Table 21

TABLE 22.

Adaptation in "minimal" medium.

Time in adapting media. Hours.	Spekker reading.	Viable count. x 10^6	Growth spekker reading. 48 hours.
0	0.116	191	0.605
6	0.082	119	0.552
19.5	0.101	0.003	0.204

TABLE 21.

Adaptation in a medium containing phosphate buffer, ammonium sulphate and glucose.

Organism.	Exp.	Time in adapting media. Hours.	Inoculum.		Growth spekker reading.	
			Spekker reading.	Viable count. $\times 10^4$	24 hours.	48 hours.
$9B_2(21)$	1.+	0	0.104	20,300	0.268	0.608
		6	0.085	12,600	0.254	0.616
		19.5	0.109	5	0.232	0.556
	2.=	0	0.166	12,200	0.182	0.257
		1.5	0.169	14,300	0.163	0.300
		19	0.170	13,000	0.136	0.357
		43	0.122	8,700	0.133	0.345
		70	0.142	4,500	0.128	0.541
$9B_2(7)$	1.+	0	0.150	21,300	0.179	0.391
		4	0.146	2,400	not readable	0.347
		7	0.129	< 10	"	0.275
		19.5	0.145	< 10	"	0.256
		28	0.122	- -	"	0.226
		49	0.133	- -	"	0.209
	2.=	0	0.165	25,500	0.190	0.466
		4	0.128	3,950	not readable	0.467
		7	0.141	< 10	"	0.044
		19.5	0.145	- -	"	not readable

+The adapting medium contained in addition magnesium sulphate, final concentration 0.004%

=The adapting medium contained only 0.1% glucose.

and show that in the medium containing 1% glucose rapid destruction of bacteria took place, irrespective of the presence of magnesium sulphate. (Destruction was very much less rapid in medium containing only 0.1% glucose). The growth after adaptation in this medium showed progressive increase in 48 but not in 24 hours.) This was surprising since this medium approximated to the "minimal" medium in which these strains grew. One obvious explanation was that the thick suspension of bacteria produced some inhibitory substances quickly and in sufficient concentration to bring about a rapid destruction of bacterial cells. In order to test the correctness of this explanation adaptation in "minimal" medium was investigated. If no such destruction occurred adaptation should take place in this medium. 3 ml. of a thick bacterial suspension of $9B_2$ (21) was mixed with 6 ml. of "minimal" medium and it was found that even in these circumstances almost complete destruction took place in 19.5 hours (see Table 22).

In all the above experiments, the criterion of adaptation was increased growth in "minimal" medium within a certain interval of time. This growth itself was a result of adaptation. It was thought that if adaptation occurred in the "adapting" medium then the delay in growth in "minimal" medium would be reduced and that growth would be greater the longer

the time of exposure, i.e. the greater the chance of adaptation in the adapting medium. In the adapting medium with no organic source of energy no adaptation occurred as indicated by growth in 24 or 48 hours. In the adapting media containing 1% glucose no conclusion could be drawn as the viable count in the inoculum became progressively smaller and any increase in growth produced by previous adaptation might be obscured by the decrease in growth due to the smaller inoculum of viable cells. With adapting media containing only 0.1% glucose and where the viable count of the inoculum did not decrease so remarkably there was a progressive increase in growth in 48 hours though not in 24 hours. This experiment has not been repeated.

The only tentative conclusion that can be drawn from the experiments so far completed is that with the criterion of "adaptation" adopted no "adaptation" without cell multiplication occurs with washed suspensions of strains $9B_2$ (21), $9B_2$ (7) and $7A_4$ (23) in a mixture of phosphate buffer and ammonium sulphate.

PART III.

THE NICOTINIC-LESS AND HISTIDINE-LESS MUTANTS OF BACT. AEROGENES A1$0_C$ AND THE POSSIBILITY OF USING THEM IN MICROBIOLOGICAL ASSAYS.

INTRODUCTION.

One of the objects of this investigation was to obtain mutant strains of Bact. aerogenes which might be suitable for use in microbiological assays of amino-acids or vitamins. Some studies on the suitability of the nicotinic-less and histidine-less mutants for this purpose are presented here.

Microbiological methods of assay have the following advantages over the chemical and physico-chemical methods. (1) They are suitable for the determination of physiologically available substances. (2) They are highly specific and highly sensitive and require a much smaller amount of the sample to be analysed. (3) Microbiological assays of amino-acids eliminate many of the laborious methods of separation necessary in the chemical analysis of proteins. (4) They are applicable in a number of instances where no other method is available or when other methods are not suitable for routine determinations. (5) They demand little expensive equipment and a similar technique can be employed in all assays. (6) They are very suitable for routine work and several estimations can be made with little more

trouble than a single one.

The disadvantages are:- (1) Unfamiliarty of a new technique which involves control of a number of factors, some of which are very imperfectly understood. (2) As only natural isomers of the amino-acids are estimated there is a danger that the unnatural isomers will be overlooked. In some cases, this specificity, of course, is advantageous. (3) With certain vitamins there is the difficulty of distinguishing between closely related substances e.g. pyridoxine, pyridoxamine and pyridoxal. (4) It is sometimes possible that unrecognised factors may alter the specificity of the test organism. (5) There is a lack of micro-organisms which can be used for the assay of certain of the amino acids. e.g. hydroxy-proline. A more complete knowledge of the metabolism of the micro-organisms employed will eliminate disadvantages (3) and (4). No. (5) may be eliminated by the study of more mutants.

Such assays have been employed extensively in the analysis of food stuffs, in metabolic studies where only small amounts of certain body fluids like blood and urine were available and in the analysis of purified proteins. A method of microbiological assay was first developed by Schopfer (1935) for vitamin B_1, using the mould Phycomyces blakesleeanus. This method had certain disadvantages and has not

been widely used. Later, Snell and Strong (1939) developed a microbiological method for riboflavin which was widely accepted for general use. Methods for most of the other members of the vitamin B-complex followed. The micro-organisms used included Lactobacilli, Proteus, dysentery bacilli, Eberthella typhosa, etc., the most extensively used organisms being the Lactobacilli. These micro-organisms required certain vitamins for their growth, the response to which in suitable media was quantitative. It was this quantitative dose-response relationship that made possible the development of the micro-biological method of assay. These same micro-organisms were later found to have well-defined nutritive requirements for amino-acids, and micro-biological methods for the determination of amino-acids were developed. Methods have also been devised for estimations of certain metals, purines, pyrimidines, haematin, and cozymase. Very excellent and extensive reviews of growth factors, their relation to micro-organisms and the assay methods which have been developed have been published recently by Peterson and Peterson, (1945); Knight (1945); Snell (1945); Barton-Wright (1945); Schweigert and Snell (1947). The assay methods need not therefore be dealt with in detail here.

All the assays using the above micro-organisms

require very complicated and expensive media and the preparation involves in addition much time and labour. If strains could be obtained which would grow in a simple medium on the addition of the particular essential nutrilite required this disadvantage would be removed. The Neurospora mutants, for example grow in a medium containing only a carbohydrate, an inorganic source of nitrogen, salts, biotin and the nutrilite required (Ryan, Beadle and Tatum, 1943). Assay methods using these mutants have been developed among others for pyridoxine (Stokes, Larson, Woodward and Foster, 1943), leucine (Ryan and Brand, 1944), lysine (Doermann, 1945), choline (Horowitz and Beadle, 1943). In using the Neurospora mutants the growth can be measured only by the laborious method of weighing the mycelium. This disadvantage has been avoided in using mutant strains of E. coli (details not yet available) which too can grow in a simple medium supplemented with the essential nutrilite required, growth being measured easily and accurately either by estimation of turbidity or of acid production.

Some of the mutant strains of Bact. aerogenes $A1O_C$ have therefore been investigated for their suitability for application in microbiological assays. These mutants grew, as shown in Part I, in "minimal" medium with the addition of the single nutrilite required and showed specificity of response. The

nicotinic-less mutants, Nos. 893 and $3A_3$, responded to nicotinic acid or its amide and to quinolinic acid (see Part I Page 48), but since quinolinic acid is not usually present in natural products it was considered that they would be suitable for the assay of nicotinic acid or nicotinamide. The specificity of response of the histidine-less mutants, Nos. 297, 506, and $9B_2$, also appeared to be satisfactory.

It was now necessary to determine whether this response to the pure nutrilite was quantitative since only if this criterion were fulfilled could the use of these mutants for microbiological methods of assay be considered.

Experience in the use of microbiological methods of assay was first obtained by courtesy of Dr. E. R. Dawson of the Distillers' Co. Ltd., in their Research and Development Laboratories at Great Burgh, Epsom, Surrey, and the opportunity was taken to carry out preliminary studies there of the growth response of the nicotinic-less mutant 893.

The dose-growth-response curve obtained was sigmoidal in shape (Fig. 8, Page 101) showing a "lag" in growth with the lower doses of nicotinic acid. It was thought that one of the reasons for this result might be the simplicity of the medium and its low buffer content. Suitable modification of the medium was attempted using the parent strain of Bact. aerogenes $A10_C$.

MODIFICATION OF THE MEDIUM.

The "minimal" medium described on page 24 was used. This was modified by addition of alternative sources of nitrogen with and without ammonium sulphate, and also by the addition of purines, xylose, and increased amounts of glucose and ammonium sulphate. The effect of increasing the phosphate content and of the addition of other buffering agents such as citrate and acetate was also studied. The growth was determined either turbidimetrically which gives a measure of the number of cells present, or acidimetrically which gives a measure of the acid production.

Experimental Procedure.

Micro-organism.

The parent strain of Bact. aerogenes $A10_C$ was obtained by single cell isolation and was then subcultured from a colony grown on "minimal" medium agar. (see Part I.)

"Minimal" (basal) medium.

The medium was prepared in double strength as follows:-

Glucose	20.0 g.
Ammonium sulphate	1.0 g.
Potassium dihydrogen phosphate	3.5 g.
$MgSo_4$, $7H_2O$	0.04g.
Glass distilled water	1000ml.

The pH was adjusted to about 7 with sodium hydroxide solution.

Inoculum.

10 ml. of sterile "complete" broth medium (page 22) was inoculated from a stock culture of the parent strain of Bact. aerogenes A10C. (Stock cultures were maintained on "complete" agar medium.) After 20 to 24 hours at 37°C. the culture was centrifuged, the bacterial sediment washed twice with 10 ml. quantities of sterile ¼ strength Ringer's solution, the third suspension in 10 ml. centrifuged for a short time at a low speed to bring down any clumps, and the uniform suspension used either as such or after further dilution.

For each assay the inoculum was freshly prepared from the stock culture.

Assay procedure.

The assays were carried out in ordinary 6"x5/8" (inner diameter) tubes. The medium (10 ml.) was pipetted into the tubes which were capped with inverted specimen tubes, sterilised by autoclaving at 10 lb. for 10 minutes, allowed to cool and inoculated as convenient with 0.05 ml. bacterial suspension to each tube. The series of tubes for each medium were set up in triplicate. The tubes were incubated in a thermostat at 37°C. for 17 to 19½ hours for the turbidimetric reading, and for one, two or three days for the acidimetric titration.

TABLE 23.

Effect on growth and acid production of the addition of xylose and purines to the basal medium.

Addition to basal medium	Turbidimetry as Spekker reading	Acidimetry as Ml. N/50 NaOH.
Nil	0.407	11.6
Xylose	0.378	12.0
Purines	0.210	9.2
Xylose + purines	0.245	8.9

The turbidimetric measurements were carried out on the Spekker absorptiometer and the results expressed as drum readings. For the acidimetric measurements, the medium was titrated with N/50, N/20 or N/10 Sodium hydroxide using bromothymol blue as indicator. The method was as follows:- One ml. of 0.04% aqueous solution of bromothymol blue was added to each tube and the contents titrated against sodium hydroxide containing 100 ml. of indicator per liter. (The indicator was added to the sodium hydroxide in order to avoid the addition of one drop per ml. of alkali during titration, as suggested by Barton-Wright and Booth (1943). Their comparator method was used in making the readings.

RESULTS.

Effect of addition of xylose and purines.

Xylose and purines were added to the basal medium in the proportions used by Barton-Wright (1944) in the medium for the assay of nicotinic acid employing Lactobacillus arabinosus i.e. to give a final concentration of 0.1% xylose and 0.001% of adenine, guanine, uracil and xanthine. Adenine, guanine and uracil were dissolved together in a small volume of water containing a few drops of acid and xanthine was dissolved in water containing a few drops of ammonia. The results, both turbidimetric and acidimetric, are given in Table 23.

TABLE 24.

Effect of addition of increased amounts of ammonium sulphate and glucose.

Addition to basal medium.	Turbidimetry Spekker readings.		Acidimetry ml. N/50 NaOH.		
	19 hr.	43 hr.	19 hr.	43 hr.	67 hr.
Exp. 1.					
Nil.		0.780			12.60
0.1%$(NH_4)_2SO_4$		0.754			12.15
0.5% " "		0.666			11.95
1% glucose		0.739			12.8
Exp. 2.					
Nil.	0.467		13.38	13.78	12.73
0.1%$(NH_4)_2SO_4$	0.415		13.68	13.43	13.13
0.16% " "	0,444		12.73	13.27	12.88

Table 23 shows that the addition of xylose did not bring about any appreciable difference in either growth or acid production and that purines, whether in the presence or absence of xylose, inhibited growth and acid production.

Effect of addition of increased amounts of ammonium sulphate and glucose.

Amounts of ammonium sulphate, 0.1%, 0.16% and 0.5% were added to the basal medium in addition to that already present. The results are given in Table 24. These show that additional ammonium sulphate did not show any appreciable difference in growth or acid production in 19 hr. although in one experiment there was a suggested increase in growth in 43 hr.

Effect of addition of other sources of nitrogen.

As the addition of extra ammonium sulphate did not show any increase either in growth in one day or in acid production, other sources of nitrogen such as the amino-acids and casein hydrolysate were tested. Lodge and Hinshelwood (1943) had shown that varying the source of nitrogen from ammonium sulphate to amino-acids giving an equivalent supply of nitrogen increased the bacterial population. Certain inexpensive and easily available amino-acids like alanine, glycine and glutamic acid were tested as the sole source of nitrogen. The amounts used contained the same amount of nitrogen as in 0.1% ammonium sulphate . The effect

TABLE 25.

Effect of addition of varied sources of nitrogen to the basal medium.

Addition to basal Medium.	Turbidimetry Spekker readings		Acidimetry. Ml. N/50 NaOH.		
	19 hrs.	43 hrs.	19 hrs.	43 hrs.	67 hrs.
Exp. 1.					
Nil.	0.407				11.6
1% asparagine	0.513				12.4
0.05% " + 0.09% glutamic acid.	0.632				13.0
Exp. 2.					
Nil.		0.780			12.6
0.1% $(NH_4)_2SO_4$		0.754			12.2
0.1% asparagine		0.821			14.1
0.18% glutamic acid		0.819			15.5
0.05% asparagine+ 0.09% glutamic acid		0.842			14.6
0.114% glycine+		0.266			16.1
0.135% alanine+		0.386			16.1
0.75% casein hydrolysate		1.413			16.4
Exp. 3.					
Nil.	0.467	0.559	13.4	13.8	12.7
0.1% $(NH_4)_2SO_4$	0.415	0.629	13.7	13.4	13.1
0.16% $(NH_4)_2SO_4$=	0.444	0.759	12.7	13.3	13.9
0.75% casein hydrolysate	1.133	1.510	18.3	18.4	17.1
0.18% glutamic acid.	0.541	0.779	15.0	16.7	15.2
0.114% glycine+	0.145	0.286	6.7	11.9	14.9
0.135% alanine+	0.172	0.397	5.5	12.8	14.5
0.18% glutamic acid+	0.212	0.385	4.8	7.2	9.7
Exp. 4.					
Nil	0.508			13.2	
0.05% lysine	0.554			14.5	
0.225% glutamic acid	0.533			16.3	
0.068% proline	0.439			12.8	
0.055% lysine + 0.225% glutamic acid	0.577			16.9	
0.05% lysine + 0.068% proline	0.527			13.5	
0.05% lysine + 0.068% proline + 0.225% glutamic acid.	0.538			17.1	
0.75% casein hydrolysate	1.107			18.4	

+The basal medium did not contain any ammonium sulphate.

=N=N in 0.75% casein hydrolysate.

of addition of these amino-acids and others (singly and in combination) and vitamin-free casein hydrolysate (Ashe Laboratories Ltd.,) in the presence of ammonium sulphate was also investigated. The results obtained in four experiments are given in Table 25.

In Experiments 1, 2, and 3 the amount of extra nitrogen was always equivalent to that in 0.1% ammonium sulphate. The amount of extra nitrogen in 0.75% vitamin-free casein hydrolysate was equivalent to that present in 0.16% ammonium sulphate. In Experiment 4, the amino-acids were added in the proportions calculated to be present in 0.75% casein hydrolysate. The percentages of the amino acids present in casein were taken from Schmidt's "Chemistry of the Amino-acids and Proteins with addendum", 1945 (page 217).

The results in Table 25 show that the addition of asparagine and glutamic acid either separately or together to the basal medium gave increased growth and acid production. Glutamic acid produced more acid than asparagine, a mixture of both giving greater increase in growth but not in acid. In the media containing glycine, alanine or glutamic acid but not ammonium sulphate, Exps. 2 and 3, both growth and acid production were slower than in the basal medium but with glycine and alanine both attained higher values in three days. The acid production increased from 12.6 with the basal medium to 16.1 with glycine and 16.1 ml.

with alanine in Exp. 2 and from 12.7 to 14.9 and 14.5 ml. respectively in Exp. 3. The acid production with glutamic acid (Exp. 3.) even in three days was much lower, 9.7 ml. compared with 12.7 ml. in the basal medium. This low rate of growth precludes the use of these amino acids as the only source of nitrogen. Addition of 0.75% casein hydrolysate produced much growth, about double that in the basal medium, while the acid production was only a third more than in the basal medium. Lysine and glutamic acid in the proportions assumed to be present in 0.75% casein hydrolysate gave increased growth and acid production. A mixture of both gave a still heavier growth although the acid production was not greater than with glutamic acid alone. Proline inhibited both growth and acid production to some extent but this inhibition was removed by the addition of lysine. A mixture of lysine, proline, and glutamic acid gave the same growth and acid production as glutamic acid. None of the sources of nitrogen, alone or in combination, gave as great a growth response as the vitamin-free casein hydrolysate.

The small increase in acid production in the presence of casein hydrolysate in contrast to the large increase in growth might possibly mean that the buffering capacity of the medium was low. Therefore further experiments were conducted using an additional buffering agent such as sodium acetate or larger amounts

TABLE 26.

The inhibitory effect of acetate and the influence of asparagine and glutamic acid on the inhibition.

Addition to Basal medium.	Turbidimetry 43 hrs. Spekker reading.	Acidimetry 3 days. Ml. N/50 NaOH.
Nil.	0.780	12.6
4% sodium acetate.	0.127	14.4
1.72% sodium chloride+	0.732	14.6
2.88% potassium acetate=	0.130	9.1
4% sodium acetate + 0.1% $(NH_4)_2SO_4$	0.101	8.6
4% sodium acetate + 0.1% asparagine	0.169	13.0
4% sodium acetate + 0.18% glutamic acid	0.040	5.2
4% sodium acetate + 0.05% asparagine + 0.09% glutamic acid.	0.065	8.7

+ Sodium ion equivalent to that in 4% sodium acetate.

= Acetate ion equivalent to that in 4% sodium acetate.

of the phosphate itself.

The effect of addition of different buffering agents.

In the preliminary experiments conducted at the laboratories of the Distillers' Co. Ltd., sodium acetate was shown to be inhibitory to the growth of the nicotinic-less mutant (893), and this was found to be the same with the parent organism too. The inhibitory effect did not seem to be due to the sodium ion but to the acetate ion, for it was found that whereas sodium chloride with an equivalent amount of sodium did not inhibit growth at all, potassium acetate with an equivalent amount of acetate inhibited growth to the same extent as sodium acetate. Addition of ammonium sulphate or asparagine did not prevent the inhibition while glutamic acid and a mixture of glutamic acid and asparagine produced a still greater inhibition. The experimental data are given in Table 26.

Since sodium acetate inhibited growth and acid production it was decided to test the effect of adding greater amounts of potassium dihydrogen phosphate itself. The phosphate was added in two (medium A), three (medium B), four (medium C), and six times (medium D) the amount present in the basal medium. At the same time, the effect of adding 0.2% ammonium sulphate, 1% glucose and a mixture of both to the modified media A, B and C was investigated. The

TABLE 27.

The effect of addition of increased amounts of phosphate and other substances on growth and acid production.

Medium.	Additions.	Turbidimetry Spekker reading. 17 hours.	Acidimetry. Ml. N/50 NaOH. 43 hours.
Exp. 1.			
Basal	nil.	0.508	5.3
A	nil.	0.519	8.5
	0.2% $(NH_4)_2SO_4$	0.550	8.1
	1% glucose	0.515	8.8
	0.2% $(NH_4)_2SO_4$+ 1% glucose	0.570	8.5
Exp. 2.			
A	nil.	0.486	9.5
	0.2% $(NH_4)_2SO_4$	0.453	9.7
	1% glucose	0.463	9.8
	0.2% $(NH_4)_2SO_4$+ 1% glucose	0.518	10.1
B	nil.	0.570	13.0
	0.2% $(NH_4)_2SO_4$	0.663	13.7
	1% glucose	0.555	14.0
	0.2% $(NH_4)_2SO_4$+ 1% glucose	0.645	14.3
C	nil.	0.691	15.6
	0.2% $(NH_4)_2SO_4$	0.720	16.8
	1% glucose	0.650	16.5
	0.2% $(NH_4)_2SO_4$+ 1% glucose	0.661	17.6
D	nil.	0.733	23.3

results of two experiments are given in Table 27.

The data given show that the additional phosphate up to six times the amount present in the basal medium increased both growth and acid production, the greatest increase taking place in the latter. Additional ammonium sulphate produced a greater increase in growth but not in acid production. Additional glucose produced a slightly greater acid production but growth was not affected.

This addition of higher amounts of phosphate and ammonium sulphate gave a satisfactory medium from the point of view of both growth and acid production. There was one disadvantage however in that media containing the greater amounts of phosphates were deep reddish brown in colour after sterilisation. Therefore, the effect of addition of another buffer, potassium or sodium citrate was investigated. Three experiments were made to study the effect of different percentages of potassium citrate and sodium citrate in media containing 0.3% ammonium sulphate and different amounts of phosphates. In one experiment, the effect of the time of incubation on acid production was also studied.

It can be seen from Table 28, Exp. I, that addition of 1% potassium citrate to all the media except D produced more growth and more acid. Inhibition of growth occured with 4% potassium citrate, this being very pronounced in Media C and D. Acid

The effect of addition of citrate to media containing different amounts of phosphate on growth and acid production.

Medium.	Additions.	Turbidimetry Spekker reading 19 hours.	Acidimetry. Ml. N/10 NaOH.	
			43 hrs.	67 hrs.
Exp. 1.				
A		0.619	3.9	
"	1% potassium citrate	0.728	7.1	
"	4% potassium citrate	0.703	12.3	
B		0.742	5.4	
"	1% potassium citrate	0.807	7.8	
"	4% potassium citrate	0.760	12.3	
C		0.766	6.5	
"	1% potassium citrate	0.849	8.9	
"	4% potassium citrate	0.472	12.7	
D		0.783	11.6	
"	1% potassium citrate	0.735	12.6	
"	4% potassium citrate	0.036	14.5	
Exp. 2.				
B		0.730	5.5	5.8
"	1% potassium citrate	0.765	8.7	7.7
"	2% potassium citrate	0.795	11.0	10.2
"	3% potassium citrate	0.758	12.2	11.3
"	4% potassium citrate	0.601	13.0	12.4
"	" " + 2% glucose	0.087+	12.1	11.3
"	3.7% sodium citrate*	0.276+	13.8	15.4
Exp. 3.				
A	2% potassium citrate	0.809	10.3	
"	4% potassium citrate	0.658	12.8	
"	1.85% sodium citrate	0.779	9.5	
"	3.7% sodium citrate	0.750	13.5	
B	2% potassium citrate	0.839	11.3	
"	4% potassium citrate	0.434	13.6	
"	1.85% sodium citrate	0.797	11.4	
"	3.7% sodium citrate	0.258	13.6	

*Molecular equivalent of 4% potassium citrate.

production was not inhibited and was maximal even in Medium A in the presence of 4% potassium citrate. For Exp. 2. Medium B was chosen as this gave maximum growth, at the same time giving least inhibition with 4% potassium citrate. The results with different percentages of citrate show that maximum growth though not acid production was obtained with 2%, and that higher percentages of citrate produced inhibition. The inhibition with 4% citrate was increased on addition of 2% glucose. An equimolecular quantity of sodium citrate produced greater inhibition of growth though not of acid production than 4% potassium citrate. Exp. 2 also showed that even in the modified media acidity was decreased on incubation for longer than two days except in the media containing sodium citrate. In Exp. 3 a comparison was made between different percentages of equimolecular quantities of potassium and sodium citrates. 2% potassium citrate gave a slightly heavier growth than 1.85% sodium citrate with both media A and B. Inhibition of growth with higher amounts of the citrates was greater with medium B. It is probable that the inhibition was due to the high concentration of buffering agents.

<u>The effect of adding other sources of nitrogen to the modified medium B.</u>

Medium B with added citrate was the most satisfactory medium as regards growth though not for acid production. As the growth could be measured

TABLE 29.

Effect of addition of other sources of nitrogen to the modified medium B.

Additions.	Turbidimetry. Spekker reading 19 hours.	Acidimetry. Ml. N/10 NaOH. 43 hours.
2% potassium citrate	0.827	11.0
" " + 0.1% asparagine	0.849	10.5
2% potassium citrate + 0.18% glutamic acid	0.663	9.4
2% potassium citrate + 0.05% asparagine + 0.09% glutamic acid	0.758	10.2
2% potassium citrate + 0.36% casein hydrolysate	1.21	8.8
1.85% sodium citrate	0.839	11.7
1.0% " "	0.841	9.1
1.0% " " + 0.1% asparagine	0.853	9.4
1.0% sodium citrate + 0.18% glutamic acid	0.663	8.3
1.0% sodium citrate + 0.05% asparagine + 0.09% glutamic acid	0.646	8.5
1.0% sodium citrate + 0.36% casein hydrolysate	1.13	8.0

turbidimetrically after one day, and as the readings themselves took less time than the titrations a medium which would give the heaviest growth was considered preferable to one which gave the highest acid production. Therefore medium B containing 0.3% ammonium sulphate and three times the phosphate present in the original basal medium (page 82) with either potassium or sodium citrate was chosen for the study of the effect of the addition of other sources of nitrogen such as asparagine, glutamic acid, and a vitamin-free casein hydrolysate. These were added in quantities which contained equivalent amounts of nitrogen. The experimental data are given in Table 29.

The addition of asparagine did not affect growth and acid production but glutamic acid seemed to inhibit both to some extent. Asparagine prevented some of the inhibition caused by glutamic acid in medium containing potassium citrate but not sodium citrate. Addition of a vitamin-free casein hydrolysate to both media gave more growth and less acid. As the addition of these amino-acids and the vitamin-free casein hydrolysate to both media did not afford any advantage, so far as both acid production and growth was concerned it was decided to use one of these media without any additional source of nitrogen. The choice between the two was determined entirely by the fact that more sodium citrate was on hand than potassium citrate.

The percentage of sodium citrate chosen was 1.85% as this gave the same degree of growth and greater acid production than 1%.

In the light of the foregoing experimental work it was concluded that addition of other sources of nitrogen did not affect growth and acid production so much as good buffering. In fact, when the medium was well-buffered only casein hydrolysate seemed to give improved growth and acid production. Purines and the amino-acid proline had an inhibitory effect while glutamic acid was inhibitory only in a well-buffered medium. Sodium acetate was inhibitory, the inhibition most probably being due to the acetate ion as potassium acetate too was inhibitory. The citrates in lower concentration gave better growth and acid production while higher concentrations were inhibitory.

The composition of the medium which was considered to be most satisfactory was as follows:-

Ammonium sulphate ..... 3.0 g.

Potassium dihydrogen phosphate ..10.5 g.

Magnesium sulphate, $MgSO_4, 7H_2O$ .. 0.04g.

Sodium citrate .....1..18.5 g.

Glucose .....20.0 g.

Glass distilled water .....1000ml.

pH adjusted to about 7.0 (6.9 to 7.1) with sodium hydroxide.

This medium was used in further studies on the mutant strains.

INVESTIGATION OF THE QUANTITATIVE RESPONSE OF THE HISTIDINE-LESS MUTANTS (506, 297, and $9B_2$) TO GRADED DOSES OF HISTIDINE.

The strains were first purified by plating on "complete" agar medium (see Part I) picking the different colonies and testing each of them in "minimal" medium. Most of the colonies from each of the strains did not grow in the "minimal" medium and after four platings one such colony from each strain was chosen which appeared to be stable. The strains 506 and 297 so chosen were found to be quite pure so far as lack of growth in "minimal" medium was concerned when retested later by the same method. The strain chosen from $9B_2$ (an adaptable mutant) grew only after one day and so could be used in quantitative investigations which could be completed in one day. The quantitative responses were studied both turbidimetrically and acidimetrically. The experimental procedure followed was the same with all the strains.

EXPERIMENTAL PROCEDURE.

Basal medium. (double the final concentration). Basal medium used was the modified "minimal" medium (see p.92) made up to 500 ml. instead of 1000 ml. Double concentration was employed as addition of the

histidine solution dilutes it further (see below). pH was checked on the pH meter, and kept always between 6.9 to 7.1.

Standard histidine solution. 50 mg. of L-histidine monohydrochloride was dissolved in glass distilled water and made up to 250 ml. This stock solution containing 200 µg./ml. could be stored in the refrigerator for a fortnight without deterioration. Fresh dilutions were prepared from this stock solution for each experiment.

Inoculum. 5 ml. basal medium + 5 ml. histidine solution (≡ 75 µg. histidine) were sterilised at a pressure of 10 lb. for 10 min. When cool this solution was inoculated from slope cultures (on complete" agar medium) and incubated at 37°C. for 21 to 24 hours. The culture was then centrifuged, washed, etc. as described on p.83. The uniform suspension obtained was diluted so that the turbidity of the inocula (read on the Spekker absorptiometer) was the same in all the experiments. For each assay the inoculum was freshly prepared from the slope culture.

Assay procedure. The procedure was the same as adopted in the investigation on the modification of medium except that graduated doses (5 ml.) of the histidine solution were first pipetted into the tubes, 5 ml. of the basal medium added, (final volume, 10 ml.) and the solution mixed well. All dose levels of the

TABLE 30.

Response to histidine of the strains 506 and 297 in modified "minimal" medium.

Turbidimetry after 17 hr. at 37°C. Spekker readings.

Histidine µg./tube.	506		297	
	Exp. 1.	Exp. 2.	Exp. 1.	Exp. 2.
0	0.019	0.053	0.054	0.093
5	0.059	-	-	-
10	0.104	0.126	0.122	0.145
20	0.182	0.186	0.188	0.190
30	0.249	0.235	0.236	0.246
40	0.304	0.287	0.289	0.285
50	0.349	0.335	0.339	0.333
75	-	0.435	0.430	0.427
100	0.548	0.516	0.503	0.503
125	-	0.571	0.580	0.580
150	-	0.636	0.645	0.635

TABLE 31.

Response to histidine of the strains 506 and 297 in modified "minimal" medium.

Acidimetry after 43 hr. at 37°C, ml. N/10 NaOH.

Histidine µg./tube.	506		297
	Exp. 1.	Exp. 2.	Exp. 1.
0	0	0	0
10	1.2	/	0.9
20	3.7	3.6	3.3
30	4.7	4.9	6.9
40	5.8	7.0	7.1
50	7.4	8.5	8.8
75	9.2	10.3	10.7
100	10.7	11.3	11.4
125	11.5	12.4	12.2
150	11.8	12.4	12.0

standards were set up in triplicate. The tubes were then capped with inverted specimen tubes, sterilised at 10 lb. for 10 min. and when cool all were inoculated with 0.05 ml. of the inoculum except for three tubes which were kept as controls. The tubes were shaken well and then incubated in the waterbath at 37°C. The turbidimetric measurements were carried out on the Spekker absorptiometer after 17 hr. and the acidimetric measurements after one or two days. The methods followed were the same as before.

RESULTS.

Strains 506 and 297.

The response to 0, 5, 10, 20, 30, 40, 50, 75, 100, 125 and 150 µg. of histidine in modified "minimal" medium of strains 506 and 297 was determined both turbidimetrically and acidimetrically. The turbidimetric measurements were made after 17 hr. the acidimetric after 43 hr.

The results are given in Tables 30 and 31. Figs. 1 and 2 show dose-response curves.

It can be seen from Table 30 that the two strains responded in the same way, the responses being remarkably alike in all four experiments. The dose-response curves shown in Fig. 1 were smooth, being practically the same for the two strains.

In the acidimetric measurements, the readings with replicates were not concordant. Perhaps these

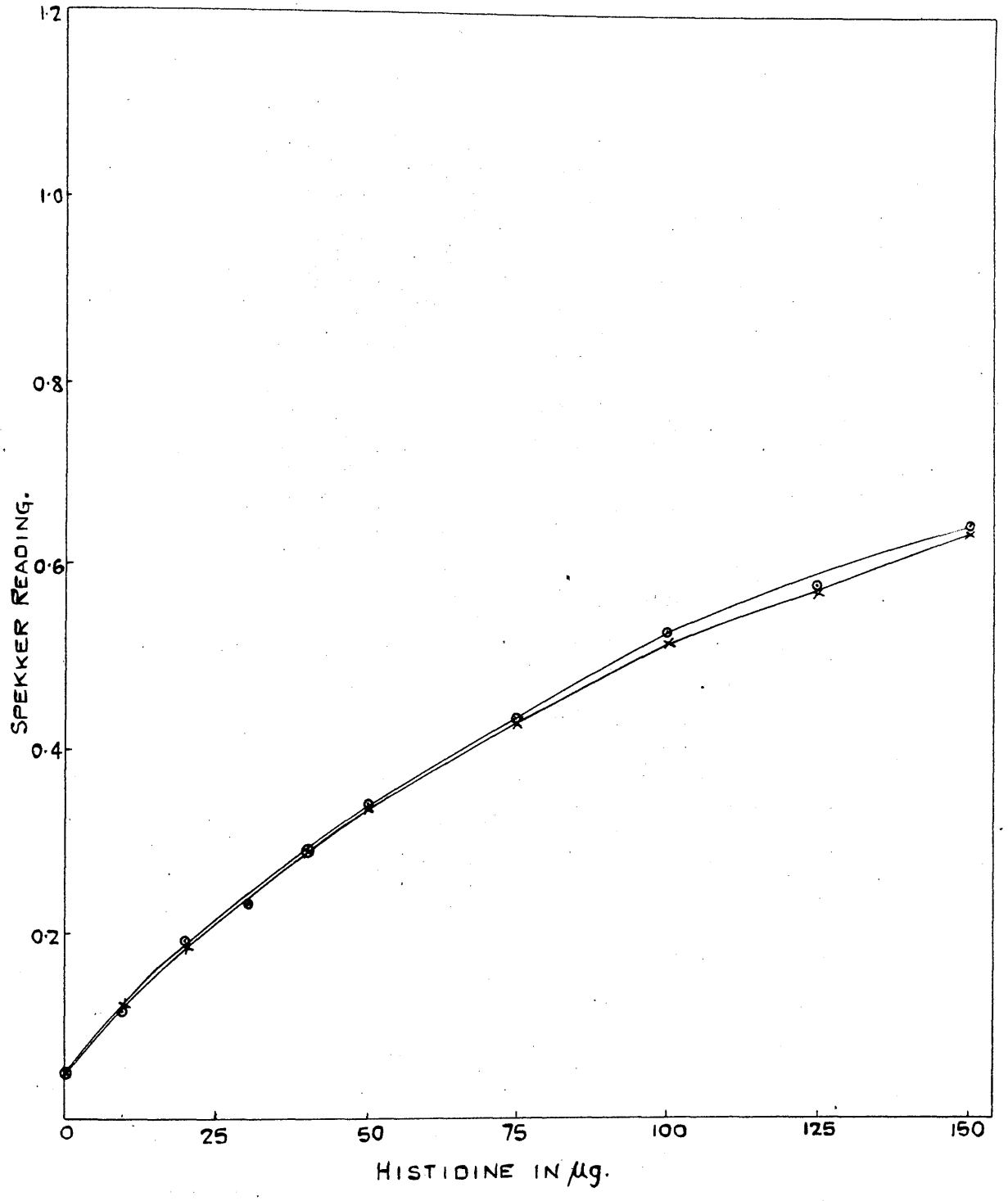


FIG. 1. DOSE-GROWTH RESPONSE CURVE OF STRAINS 506 AND 297 WITH HISTIDINE.

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS. AT 37°C.

×——× STRAIN 506

⊙——⊙ STRAIN 297.

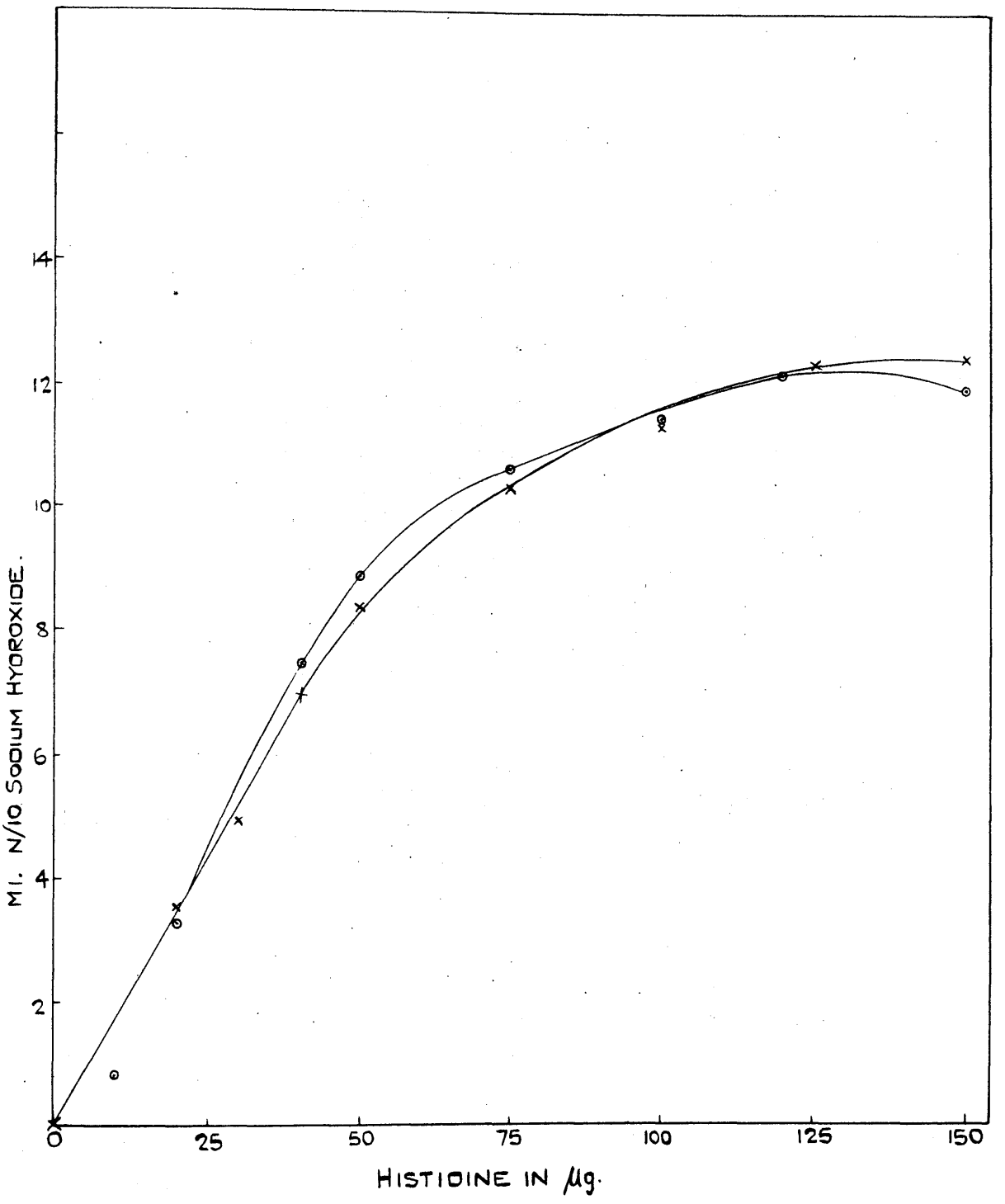


FIG. 2. DOSE-ACID PRODUCTION RESPONSE CURVE OF STRAINS 506 AND 297.

ACIDIMETRIC MEASUREMENTS AFTER 43 HRS. AT 37°C.

×—× STRAIN 506

⊙—⊙ STRAIN 297

TABLE 32.

Response to histidine of the strain $9B_2$ in modified "minimal" medium.

Histidine µg/tube	Turbidimetry. Spekker reading. 17 hrs. at 37°C.		Acidimetry 19 hrs. at 37°C. Ml. N/10 NaOH.
	Exp. 1	Exp. 2	Exp. 1
0	0.040	0.044	0
0.5	-	0.151	1.2
1.0	-	0.205	4.1
2.0	-	0.271	5.6
4.0	-	0.371	6.9
6.0	-	0.399	7.7
10.0	0.493	0.599	8.9
15.0	-	0.640	10.2
20.0	0.703	0.755	10.7
30.0	0.756	0.885	11.4
40.0	0.810		
50.0	0.807		
75.0	0.826		
100.0	0.896		
125.0	0.901		
150.0	0.927		

discrepancies could be removed by titrating within 24 hours but this has not yet been investigated. The dose-response curves with both strains as shown in Fig. 2. are almost identical.

The results in Tables 30 and 31 show that the two strains 506 and 297 could be assumed to be identical and further experiments were conducted only with the strain 506. ~~only~~.

Strain $9B_2$.

The response to 0, 0.5, 1, 2, 4, 6, 10, 15, 30, 40, 50, 75, 100, 125, and 150 µg. histidine in modified "minimal" medium of strain $9B_2$ was determined both turbidimetrically and acidimetrically. The turbidimetric measurements were made after 17 hr. and the acidimetric after 19 hr.

The results are given in Table 32 and plotted in Fig. 3. The strain $9B_2$ seemed to require much less histidine than the others, 10 µg. producing as much growth with strain $9B_2$ as 125 µg. with strains 506 and 297. (As little as 0.5 µg. of histidine gives a satisfactory response.) Both turbidimetric and acidimetric measurements gave concordant results with replicates. It is interesting to speculate that this sensitivity of the strain to histidine may be due to its adaptability.

Response to histidine of the strains 506 and $9B_2$ in the original and modified "minimal" media.

It was important to determine how the behaviour

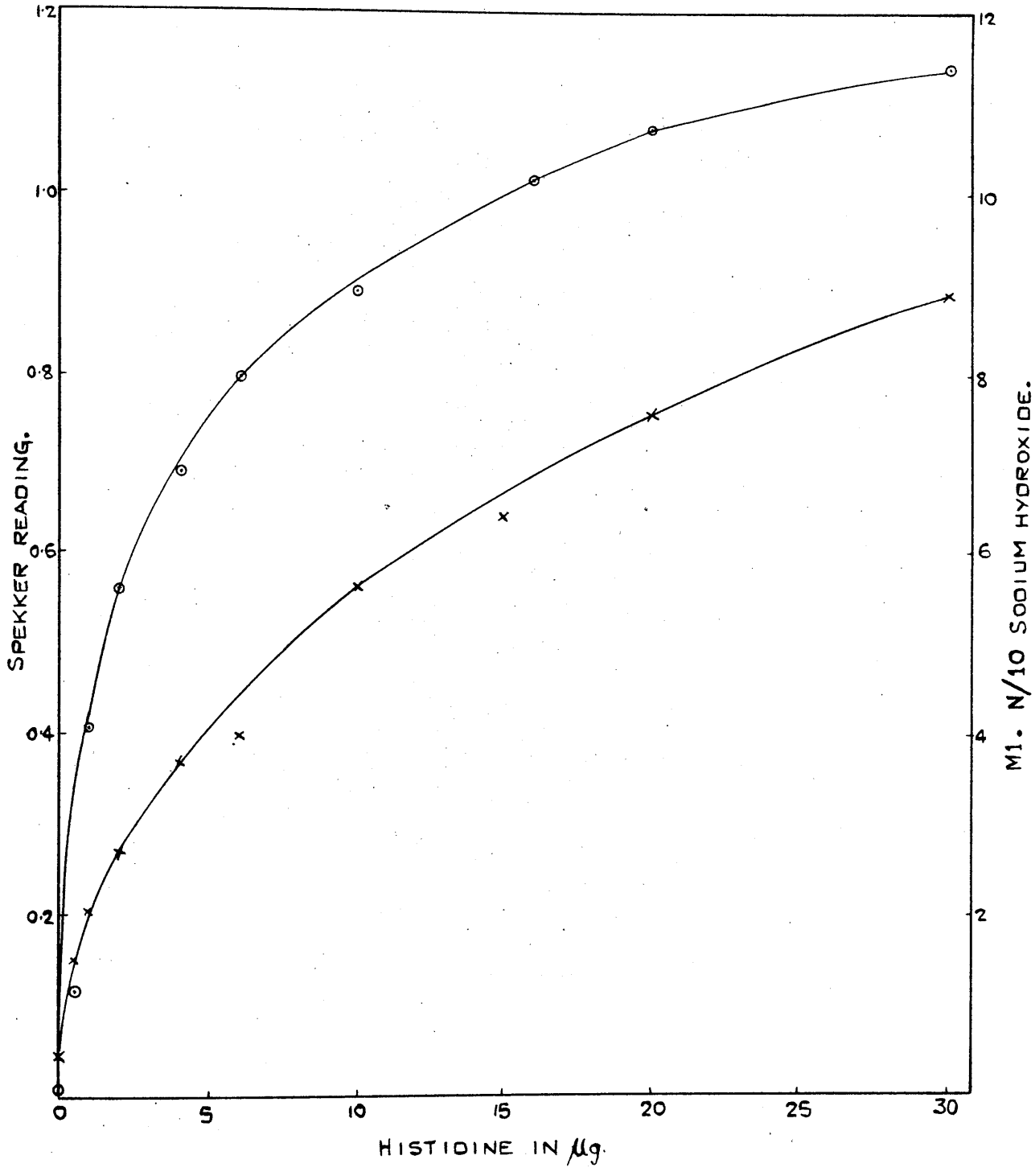


FIG. 3. DOSE-RESPONSE CURVE OF STRAIN 9B2 WITH HISTIDINE

×——× TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS. AT 37°C. SPEKKER READING.

⊙——⊙ ACIDIMETRIC MEASUREMENTS AFTER 19 HRS. AT 37°C. Ml. OF N/10 SODIUM HYDROXIDE.

TABLE 33

Response to histidine of the strains 506 and $9B_2$ in the original and modified "minimal" medium.

Turbidimetry. 17 hours at 37°C. Spekker readings.

Histidine µg/tube.	506		$9B_2$	
	Original.	Modified.	Original.	Modified.
0	0.026	0.029	0.024	0.027
0.5			0.044	0.119
1.0			0.073	0.186
2.0			0.120	0.266
4.0			0.220	0.355
6.0			0.268	0.425
10.0	0.082	0.114	0.331	0.540
15.0			0.325	0.629
20.0	0.127	0.185	0.350	0.718
30.0	0.175	0.235	0.450	0.759
40.0	0.235	0.294		
50.0	0.287	0.333		
75.0	0.364	0.440		
100.0	0.397	0.536		
125.0	0.409	0.596		
150.0	0.413	0.658		

of the strains would be affected by modifications of the medium. Therefore the responses of the strains 506 and $9B_2$ to different doses of histidine in the original and modified "minimal" media were determined turbidimetrically. The composition of the original media is given on p.38. The results are shown in Table 33 and plotted in Figs. 4 and 5.

In the original medium the response was lower and the dose-response curve shows that the bend in the curve occurs earlier, the maximum growth being reached at a lower dose level of histidine. With 506, maximum growth was reached with about 100 µg. histidine while in modified medium even with 150 µg. maximum growth was not reached. With $9B_2$, the curve was irregular for doses greater than 15 µg. While in the modified medium the curve was smooth up to the 30 µg. level.

CONCLUSIONS.

The results show that the histidine-less mutant strains 506, 297 and $9B_2$ responded quantitatively to graded doses of histidine, the "adaptable" strain $9B_2$ being more sensitive. The strains 506 and 297 responded in the same manner and so have been assumed to be the same. Turbidimetric measurements were more satisfactory than the acidimetric measurements made in two days. A well-buffered medium gave better and more reglular results than one that was poorly buffered.

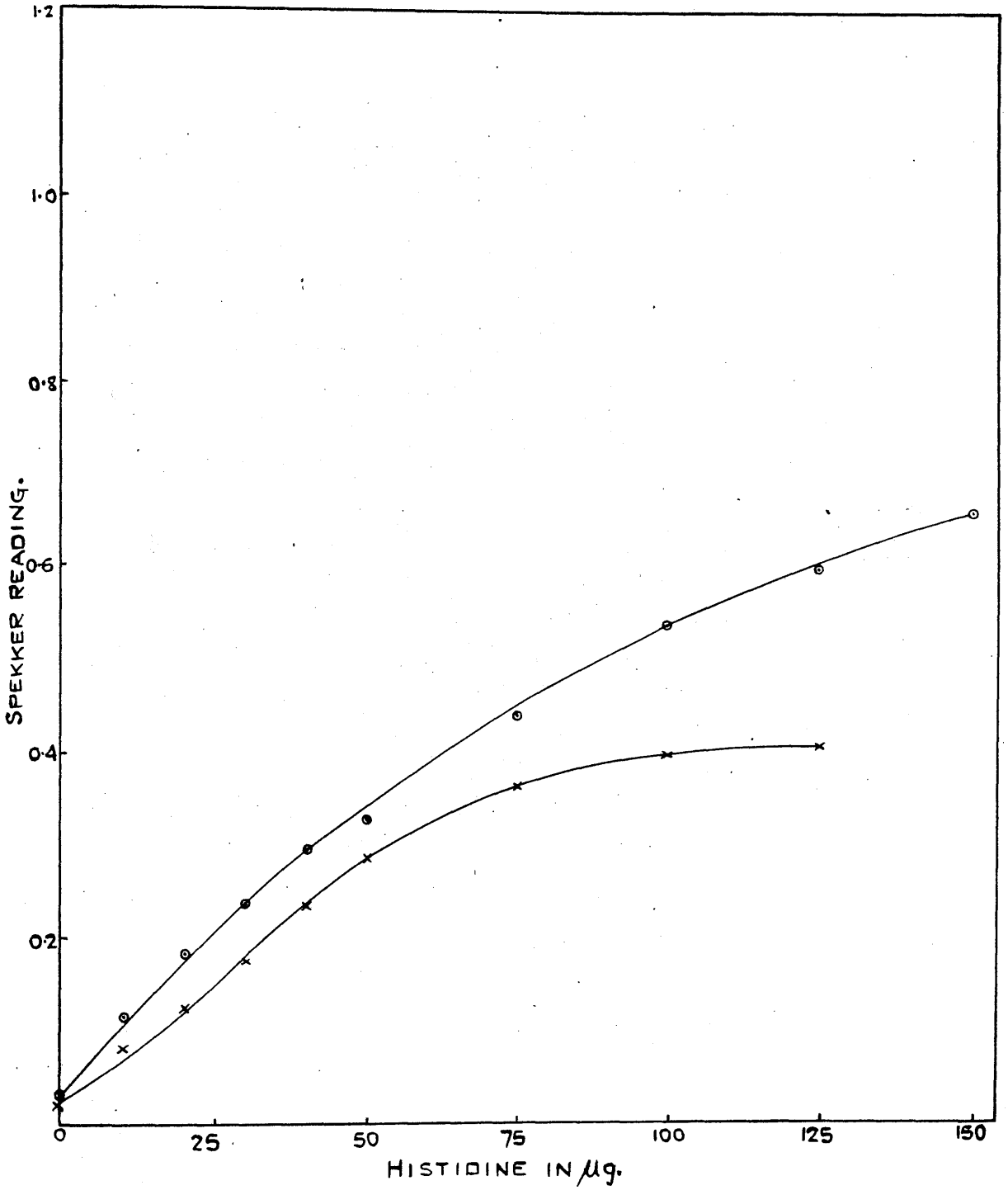


FIG. 4. DOSE-GROWTH RESPONSE CURVE OF STRAIN 506 WITH HISTISDINE TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS. AT 37°C.

×——× ORIGINAL "MINIMAL" MEDIUM.

⊙——⊙ MODIFIED "MINIMAL" MEDIUM.

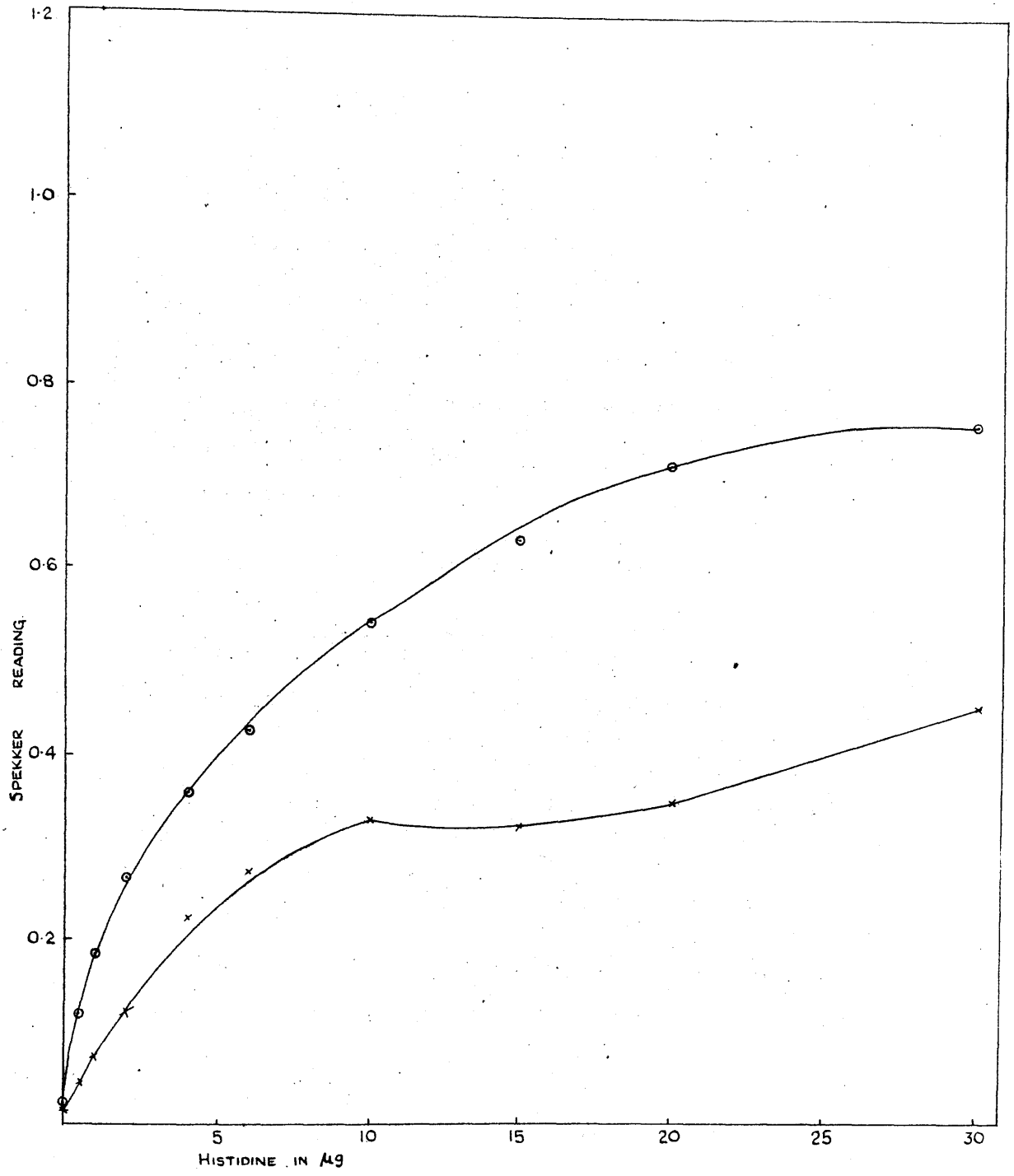


FIG 5. DOSE - GROWTH RESPONSE CURVE OF 9B2 TO HISTIDINE
TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS AT 37°c.

×——× ORIGINAL "MINIMAL" MEDIUM.

⊙——⊙ MODIFIED "MINIMAL" MEDIUM.

These strains could be considered to be satisfactory for application in microbioligical assays of histidine so far as quantitative responses to graded doses of histidine were concerned. It remains to be seen whether in an assay of histidine present in proteins and food stuffs the response would be still so quantitative. The amino-acids and other substances present at the same time may have a non-specific effect on the response. It might be necessary to modify the basal medium further to overcome this effect.

INVESTIGATION OF THE SUITABILITY OF THE NICOTINIC-LESS MUTANTS, 893 AND $3A_3$, FOR APPLICATION IN MICROBIOLOGICAL ASSAYS.

The nicotinic-less mutant 893 was a strain obtained from the parent culture by spontaneous variation. $3A_3$ was obtained after exposure to X-rays.

Part of the work presented here on the response of the strain 893 to nicotinic acid was conducted at the Research and Development Laboratories of the Distillers Co., Ltd., at Great Burgh, Epsom, by arrangement with Dr. E. R. Dawson.

Experimental Procedure.

Basal medium (double the final concentration).

This was the same as used in the investigations on the histidine-less mutants. (p.92). The H-ion concentration

was checked on the pH meter and except where some other value is mentioned kept between 6.9 and 7.1.

Standard solutions.

(a) Nicotinic acid solution. 50 mg. were dissolved in glass-distilled water and made up to 250 ml. This stock solution containing 200 µg./ml. could be stored in the refrigerator for a fortnight without deterioration. Fresh dilutions were prepared from this stock solution for each experiment.

(b) Nicotinamide solution. 50 mg. of the amide were dissolved in glass distilled water and made up to 250 ml. This solution, containing 200 µg./ml. was less stable than the nicotinic acid solution. Fresh dilutions were prepared from the stock solution, for each experiment.

(c) Quinolinic acid solution. 68 mg. quinolinic acid were dissolved in glass distilled water and made up to 250 ml. This contained a molecular equivalent of 200 µg./ml. of nicotinic acid. Fresh dilutions were prepared from this stock solution for each experiment.

Inoculum.

5 ml. basal medium + 5 ml. nicotinic acid solution (≡0.5 µg. nicotinic acid) were sterilised at 10 lb. for 10 minutes. When cool this was inoculated from slope cultures (on "complete" agar medium) and incubated at 37°C for 21 to 24 hours. The culture was then

TABLE 34.

Effect of incubation temperature on response to nicotinic acid of strain 893 in original "minimal" medium.

Acidimetry after 3 days at 37° and 30°C.

Nicotinic acid µg./tube	Ml. N/50 NaOH.	
	37°C.	30°C.
0.0	0	0
0.1	5.9	0
0.2	7.8	4.8
0.3	9.5	6.0
0.4	10.8	6.5
0.5	10.1	7.9
0.6	8.8	8.2

centrifuged, washed, etc. as given on p.83. The uniform suspension obtained was diluted so that the turbidity of the inoculum (read on the Spekker absorptiometer) was the same in all the experiments unless otherwise mentioned. 0.05 ml. was inoculated into each tube. For each assay the inoculum was freshly prepared from the slope culture (subcultured every fortnight).

Assay.

The procedure was the same as that adopted for investigations on the histidine-less mutants and therefore is not given in detail here. The turbidimetric measurements were carried out on the Spekker absorptiometer usually after 17 hr. incubation and acidimetric measurements after two or three days.

The methods followed were the same as before.

Results.

Effect of incubation temperature on response of strains 893 to nicotinic acid in original "minimal" medium.

The original "minimal" medium as given on p.82 was prepared in double strength. The response to 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µg. nicotinic acid after three days incubation at 37°C or 30°C was determined acidimetrically by titration with N/50 sodium hydroxide. The results are given in Table 34 and in the dose-response curve in Fig. 6. The results show that at 30°C. growth was definitely slower than at 37°C.

All the following experiments were, therefore, conducted at 37°C.

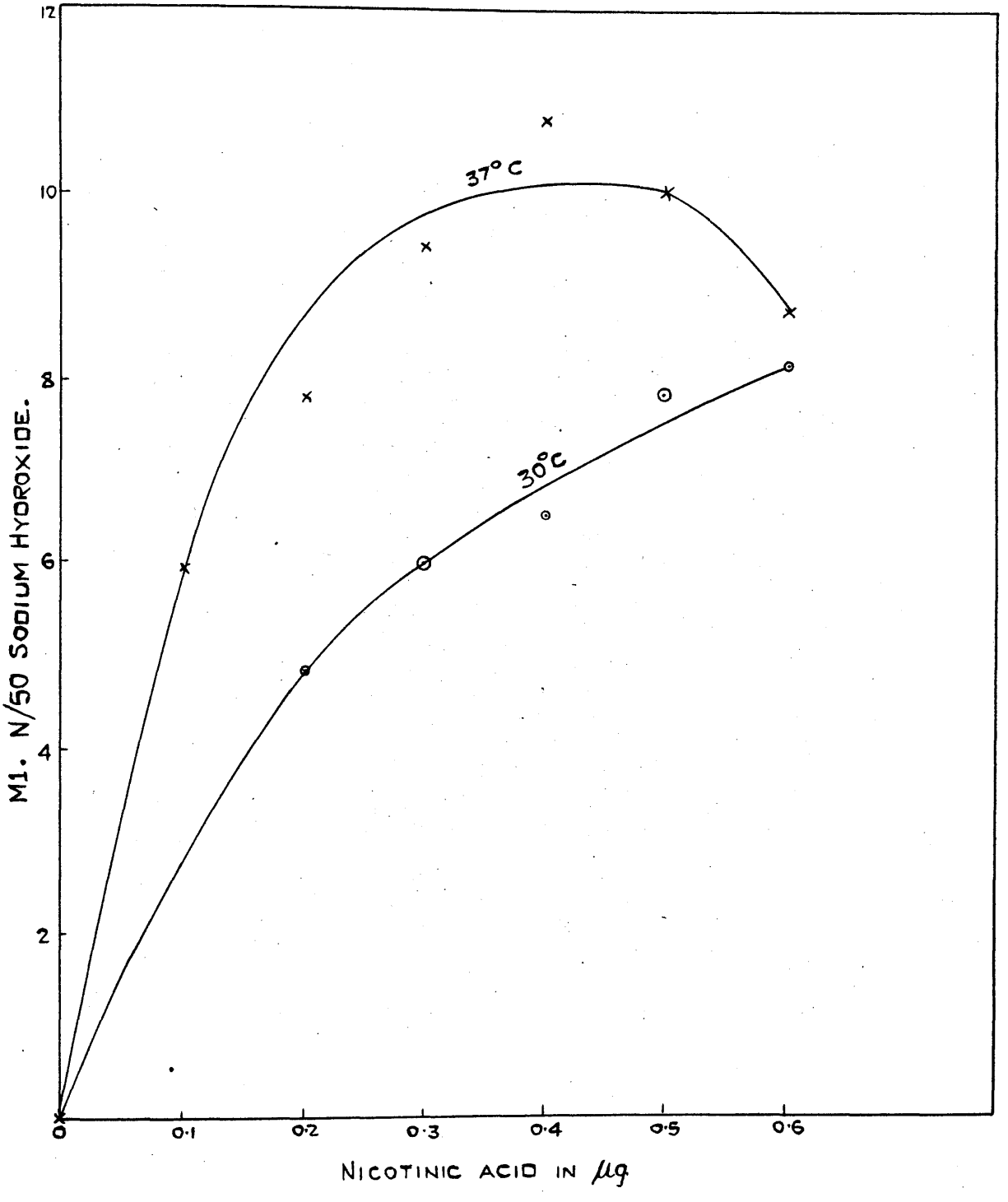


FIG. 6. DOSE-RESPONSE CURVE OF STRAIN 893 WITH NICOTINIC ACID AT 37°C. AND 30°C. IN ORIGINAL "MINIMAL" MEDIUM.

ACIDIMETRIC MEASUREMENTS AFTER 3 DAYS.

TABLE 35.

Response to nicotinic acid of strain 893 in various media.

Acidimetry after 2 days at 37°C.

Nicotinic acid µg./tube.	Ml. N/50 NaOH.			
	Original "minimal" medium.	Original "minimal" medium + casein hydrolysate	Original "minimal" medium + sodium acetate.	Original "minimal" medium + acetate + casein hydrolysate.
0	0	1.1	0	0.2
0.02	0.20	2.2	0	0.2
0.04	0.95	2.5	0	0.5
0.1	4.95	6.2	0.8	3.4
0.2	5.80	6.9	2.4	4.3
0.4	7.80	10.6	2.2	5.0
1.0	8.30	10.0	10.1	2.9

TABLE 36.

Response to nicotinic acid of strain 893 in various media.

Turbidimetry after 17 hr. at 37°C. Spekker readings.

Nicotinic acid µg./tube.	Original "minimal" medium.	Original "minimal" medium + casein hydrolysate.	Original "minimal" medium + acetate.	Original "minimal" medium + acetate + casein hydrolysate.
0	0.003	0.008	0.008	0.003
0.02	0.005	0.009	0.014	0.004
0.04	0.004		0.0	0.016
0.10	0.019	0.009	0.0	0.018
0.20	0.098	0.126	0.015	0.023
0.40	0.443	0.516	0.063	0.160
1.0	0.493	0.666	0.091	0.583

Response to nicotinic acid of strain 893 in original "minimal" medium alone and with certain additional substances.

The dose-response was determined in the original "minimal" medium with

(a) no addition.

(b) vitamin-free casein hydrolysate (0.3%)

(c) sodium acetate (1.6%)

(d) casein hydrolysate (0.3%) and sodium acetate (1.6%)

Doses of 0.02, 0.04, 0.1, 0.2, 0.4 and 1.0 µg. nicotinic acid were used. For acidimetric measurements one tube at each level was taken and for turbidimetric two tubes at each level.

The titration figures are given in Table 35 and are plotted in Fig. 7. Sodium acetate inhibited the growth but the inhibitory effect appeared to be removed or masked to a great extent by the addition of casein hydrolysate. The response was greater when casein hydrolysate only was added than in the original "minimal" medium itself.

The response measured by turbidimetric means (Table 36, Fig. 8) was similar to that using acidimetric methods (Table 35, Fig. 7) with one important difference. The measurement of turbidity gave a sigmoidal curve in that at low concentrations of nicotinic acid there was very little growth while at higher concentrations rapid

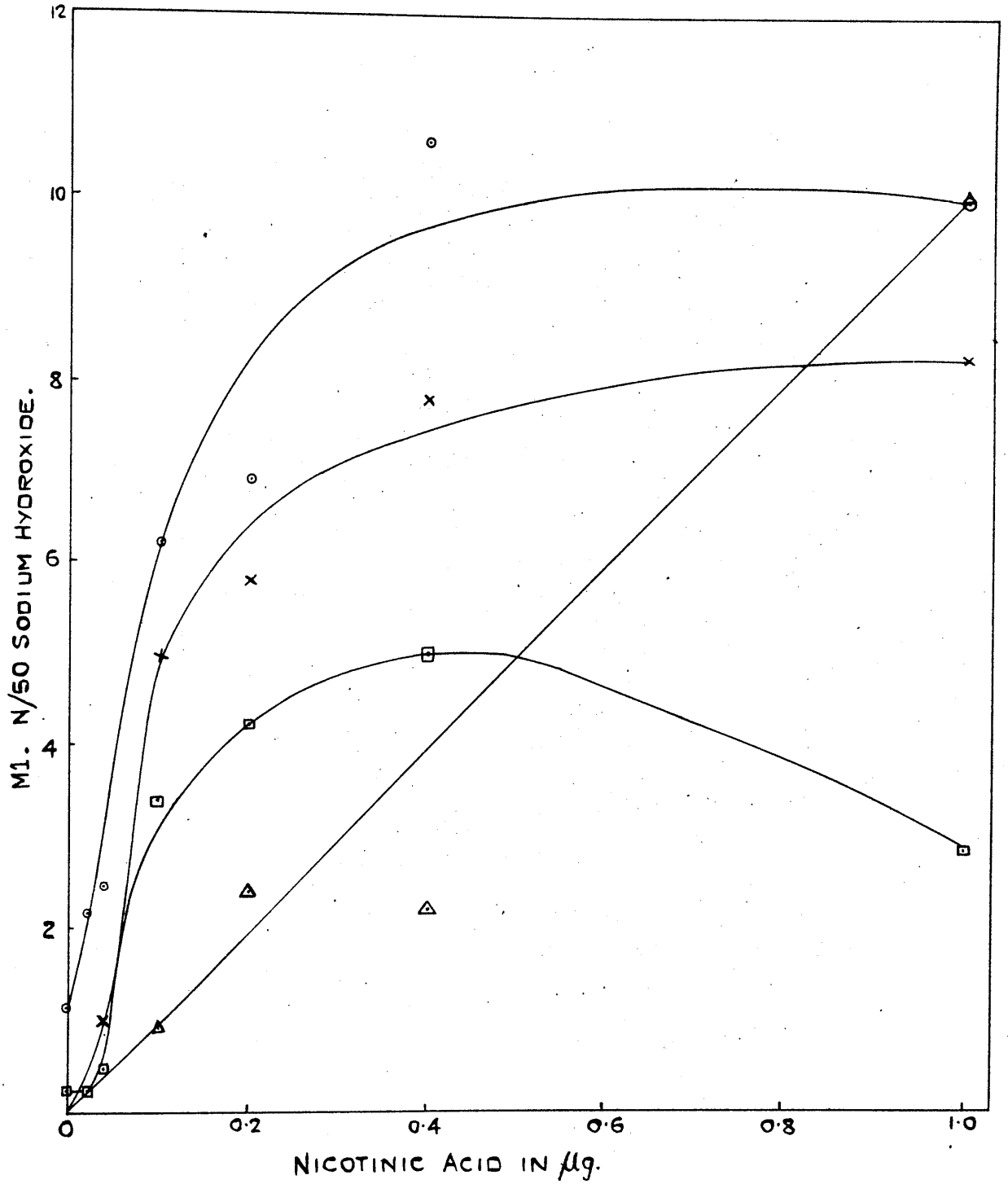


FIG. 7. DOSE-RESPONSE CURVE OF STRAIN 893 WITH NICOTINIC ACID IN VARIOUS MEDIA.

ACIDIMETRIC MEASUREMENTS AFTER 2 DAYS AT 37°C.
×—× ORIGINAL "MINIMAL" MEDIUM (O.M.M.)
⊙—⊙ O.M.M. + CASEIN HYDROLYSATE.
△—△ O.M.M. + SODIUM ACETATE.
⊡—⊡ O.M.M. + SODIUM ACETATE + CASEIN HYDROLYSATE.

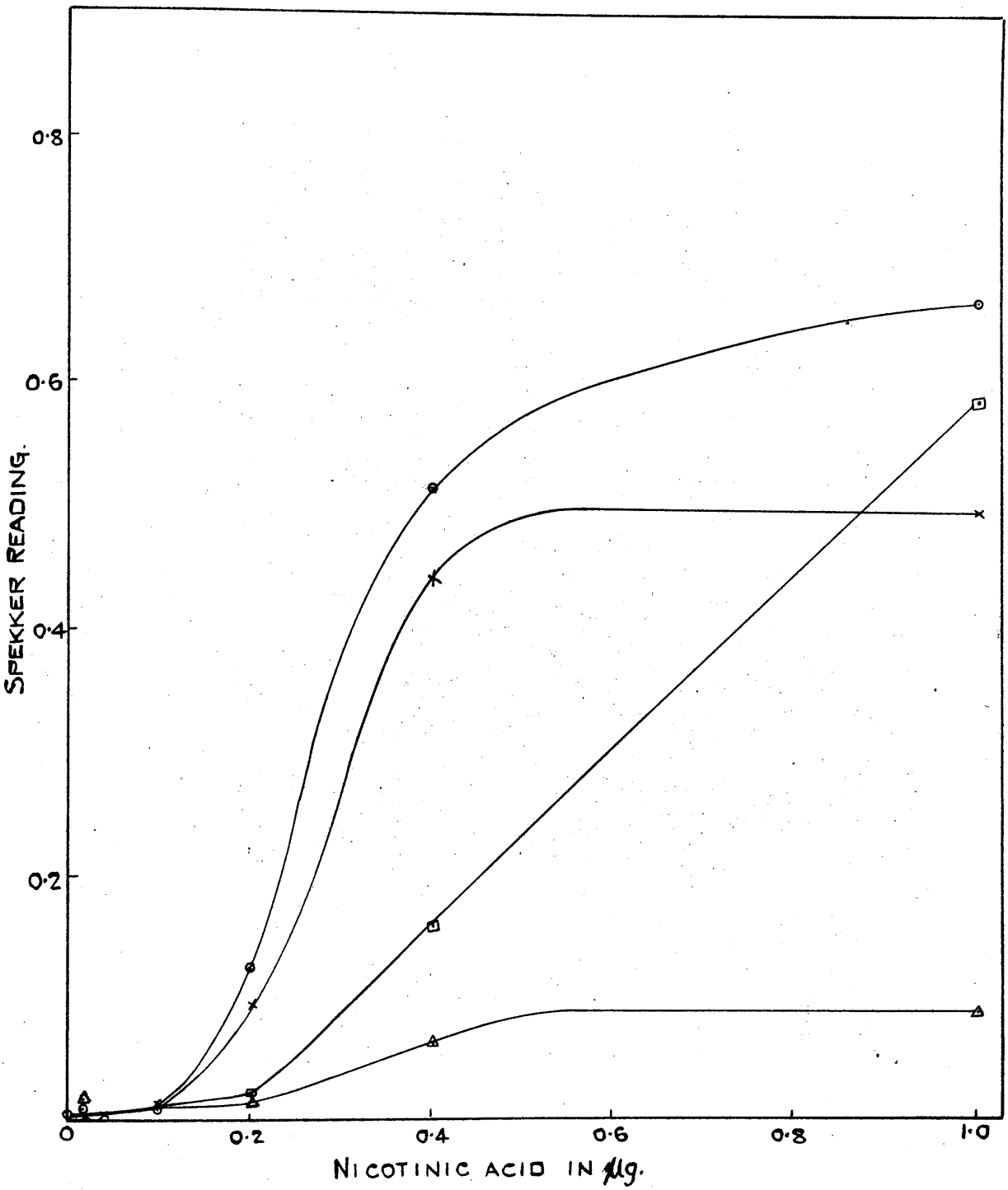


FIG. 8. DOSE-GROWTH RESPONSE CURVE OF STRAIN 893 WITH NICOTINIC ACID IN VARIOUS MEDIA.

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS. AT 37°C.
×—× ORIGINAL "MINIMAL" MEDIUM. (O.M.M.)
⊙—⊙ O.M.M. + CASEIN HYDROLYSATE.
△—△ O.M.M. + SODIUM ACETATE.
⊡—⊡ O.M.M. + ACETATE + CASEIN HYDROLYSATE.

TABLE 37.

Response to nicotinamide of strain 893 in original "minimal" medium.

Turbidimetry after 17 hr. at 37°C. Spekker readings.

Concentration. µg./tube.	Exp. 1.		Exp. 2.	
	Nicotinic acid.	Amide	Nicotinic acid.	Amide.
0.05		0.087		0.112
0.10	0.007	0.200	0.003	0.229
0.15	0.014	0.237		0.289
0.20	0.086	0.301	0.005	0.315
0.30	0.240	0.350	0.045	0.385
0.4			0.148	
0.5			0.314	
0.6	0.410		0.388	

growth occured. At the lower levels of nicotinic acid growth was not proportional to the concentration of nicotinic acid. This "lag" in growth was confirmed in further assays but the concentration of nicotinic acid required to overcome the "lag" period varied with each experiment. By acidimetric methods this "lag" did not appear.

Response to nicotinamide in original "minimal" medium.

Experiments were conducted to find whether the response to nicotinamide was the same as to that to nicotinic acid. The results of two experiments with both nicotinamide and nicotinic acid are given in Table 37.

The response to nicotinamide was similar in both experiments and differed from the response to the nicotinic acid in showing no "lag" period although the middle parts of the dose-response curve (Fig. 9) were more or less parallel.

These preliminary experiments have established the occurrence of a "lag" phase in the growth response to nicotinic acid. This may have resulted from the simplicity of the medium, its low buffer action or an unsuitable pH, or other factors.

Before further studies of the nicotinic-less mutants were undertaken, the medium was modified during the examination of the histidine-less mutant as described in the section "Modification of the Medium" in p. 92.

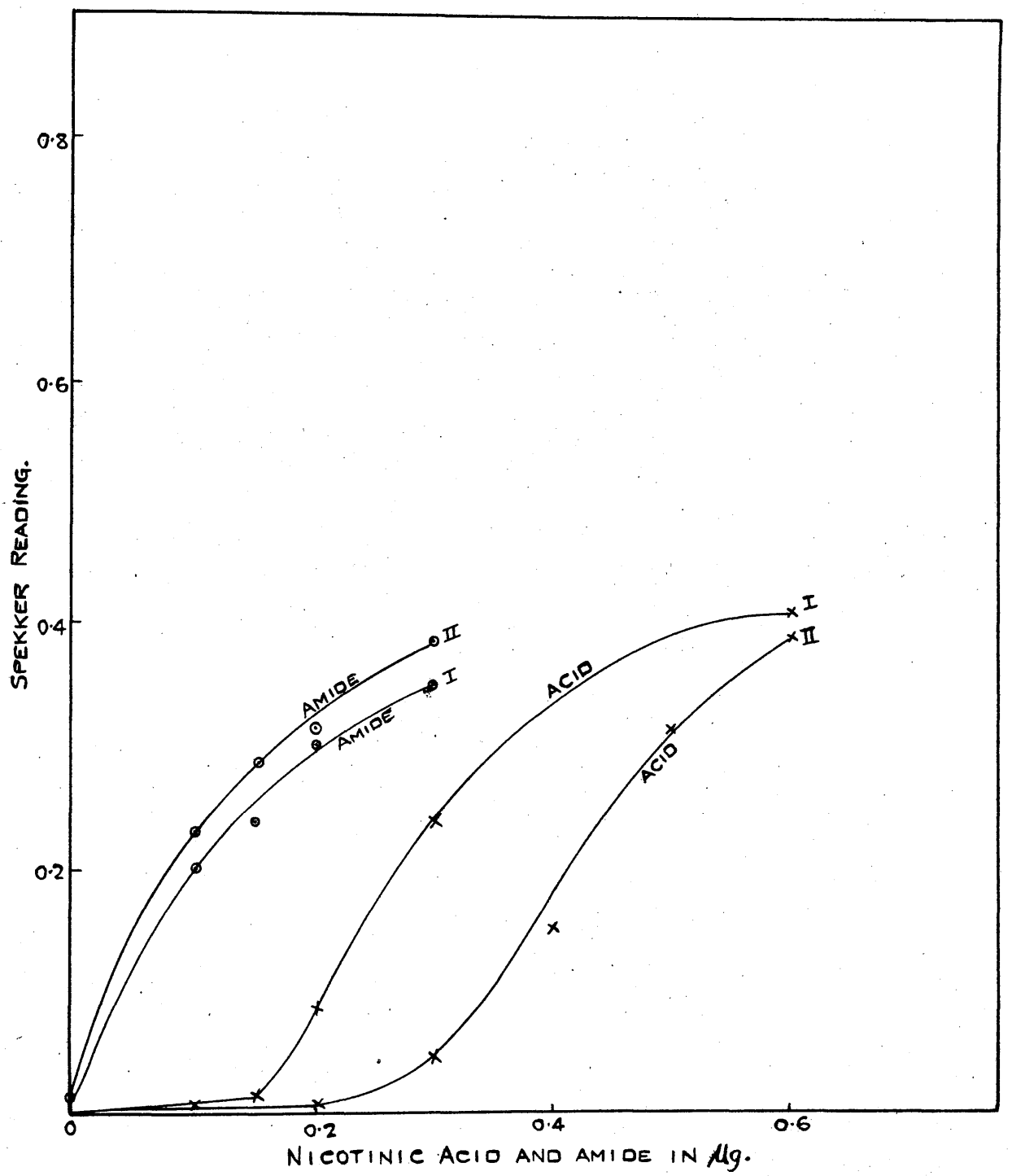


FIG. 9. DOSE-GROWTH RESPONSE CURVE OF STRAIN 893 WITH NICOTINIC ACID AND NICOTINAMIDE IN ORIGINAL "MINIMAL" MEDIUM.

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS. AT 37°C.

TABLE 38.

Response to nicotinic acid of strains 893 and 3A$_3$ in original and modified "minimal" media.

Acidimetry after 2 days at 37°C. Ml. N/10 NaOH.

Nicotinic acid µg./tube.	893		3A3	
	Medium.		Medium.	
	Original	Modified	Original	Modified
0	0	0	0	0
0.5	0.2	0.1	0.4	0.3
0.1	0.9	0.7	1.3	1.3
0.2	1.5	1.3	1.9	2.3
0.3	1.8	3.1	2.3	3.8
0.4	2.5	4.6	2.5	5.3
0.5	2.6	6.4	2.6	6.7
0.6	2.6	7.7	2.6	8.1
0.8	2.8	9.6	2.7	9.8
1.0	2.8	11.2	2.6	10.8

TABLE 39.

Response to nicotinic acid of strains 893 and 3A3 in original and modified media.

Turbidimetry after 17 hr. at 37°C. Spekker reading.

Nicotinic acid µg./tube.	893		3A$_3$	
	Medium.		Medium.	
	Original	Modified	Original	Modified
0	0.039	0.073	0.043	0.085
0.05	0.048	0.073	0.045	0.093
0.1	0.062	0.093	0.060	0.117
0.2	0.075	0.172	0.100	0.261
0.3	0.116	0.309	0.200	0.391
0.4	0.289	0.434	0.323	0.497
0.5	0.370	0.562	0.389	0.555
0.6	0.374	0.606	0.402	0.603
0.8	0.451	0.677	0.382	0.683
1.0	0.435	0.720	0.378	0.741

This modified "minimal" medium finally adopted contained three times the original concentration of phosphate and of ammonium sulphate and in addition sodium citrate. The composition is given on p. 92. This modified medium was used as the basal medium in the following studies of the nicotinic-less mutants 893 and $3A_3$.

Response to nicotinic acid of strains 893 and $3A_3$ in original and modified "minimal" media.

The response to 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 µg. of nicotinic acid of both the strains in the original and modified "minimal" media was determined acidimetrically and turbidimetrically.

The acidimetric results given in Table 38 and Fig. 10 show that the maximum response of both the strains was much greater in the modified than in the original medium. In the latter, the maximum response was reached with 0.4 µg. while in the modified medium the response continued to increase up to 1 µg. nicotinic acid. Both strains gave similar responses in each of the two media.

The turbidimetric results given in Table 39 and Fig. 11 also show that a greater growth response was obtained in the modified than in the original medium. The response was similar with both strains. As before there was a "lag" in the growth response to lower concentrations of nicotinic acid, the concentration at which the "lag" disappeared being lower in the modified

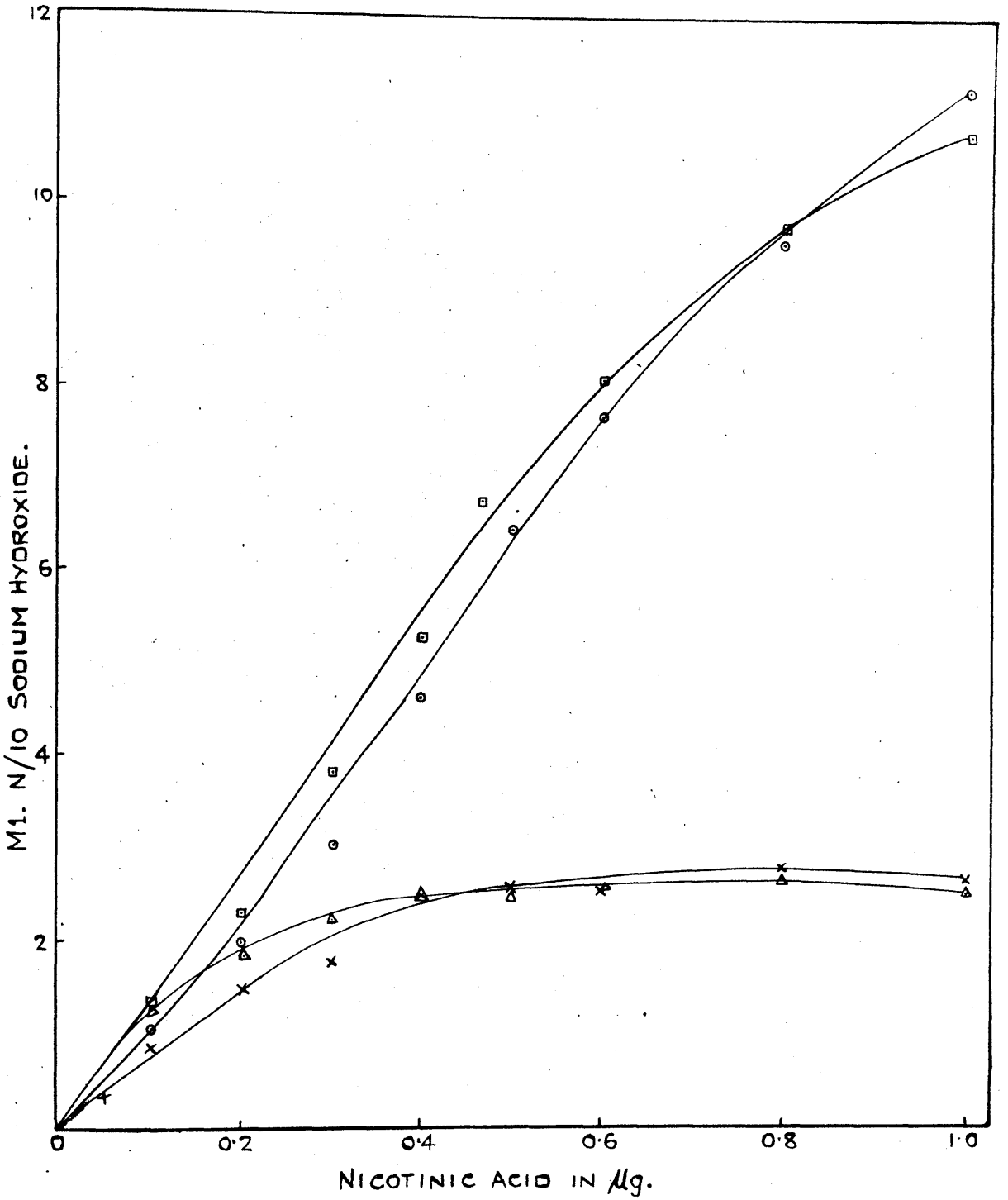


FIG. 10. DOSE-RESPONSE CURVE OF STRAINS 893 AND 3A3 WITH NICOTINIC ACID IN ORIGINAL AND MODIFIED "MINIMAL" MEDIA.

ACIDIMETRIC MEASUREMENTS AFTER 2 DAYS AT 37°C.

×—× STRAIN 893 IN O.M.M.
⊙—⊙ " " " M.M.M.
Δ—Δ STRAIN 3A3 IN O.M.M.
⊡—⊡ " " " M.M.M.

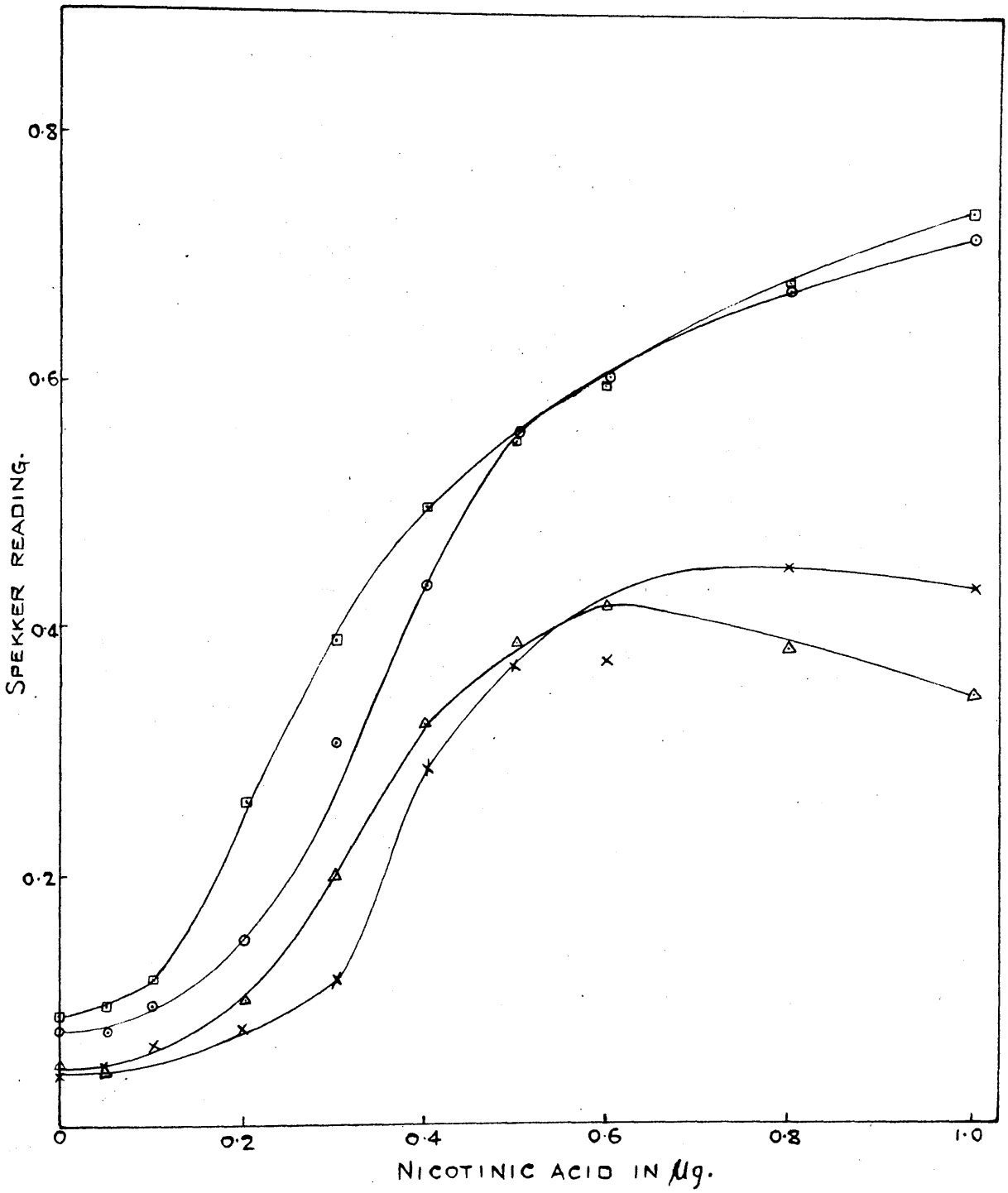


FIG. 11. DOSE-GROWTH RESPONSE CURVE OF STRAINS 893 AND 3A3 WITH NICOTINIC ACID IN ORIGINAL AND MODIFIED "MINIMAL" MEDIA.

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS. AT 37°C.

×——× STRAIN 893 IN O.M.M.
⊙——⊙ " " " M.M.M.
△——△ STRAIN 3A3 " O.M.M.
⊡——⊡ " " " M.M.M.

TABLE 40.

Response to nicotinic acid of strains 893 and $3A_3$.

Turbidimetry after 17 hr. at 37°C. Spekker reading.

Nicotinic acid µg./tube.	893			$3A_3$		
	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3
0	0.053	0.034	0.026	0.067	0.027	0.013
0.1	0.059	0.035	0.018	0.078	0.050	0.020
0.2	0.080	0.054	0.027	0.116	0.137	0.052
0.3	0.109	0.189	0.041	0.177	0.295	0.093
0.5	0.393	0.487	0.442	0.518	0.608	0.460
0.75	0.853	0.606	0.660	0.908	0.653	0.667
1.0	0.774	0.650	0.737	0.814	0.747	0.758
1.5	0.828	0.766	0.816	0.848	0.840	0.820

TABLE 41.

Response to nicotinic acid of strain 893 in basal medium with and without casein hydrolysate.

Turbidimetry after 17 hr. at 37°C. Spekker reading.

Nicotinic acid µg./tube.	Exp. 1.		Exp. 2.	
	Medium.		Medium.	
	Basal	+casein hydrolysate	Basal	+casein hydrolysate
0	0.043	0.050	0.031	0.016
0.1	0.041	0.058	0.018	0.021
0.2	0.073	0.080	0.027	0.042
0.3	0.156	0.100	0.041	0.058
0.5	0.496	0.350	0.442	0.126
0.75	0.655	0.909	0.660	0.386
1.0	0.709	1.080	0.737	0.878
1.5	0.790	1.143	0.810	1.083

than in the original medium.

The concentrations of nicotinic acid required to prevent the "lag" in growth rate varied as shown for three experiments in Table 40 and plotted for strain 893 in Fig. 12. The curves for strain $3A_3$ showed a similar variation. The inoculum was adjusted to the same turbidity in each experiment.

Further experiments on the response to nicotinic acid were conducted in modified "minimal" medium only using strain 893 as the two strains were found to behave similarly.

Response to nicotinic acid of strain 893 in basal medium plus casein hydrolysate.

It was considered important to determine whether further enrichment of the medium by the addition of vitamin-free casein hydrolysate would affect the "lag". Addition of this substance to the medium had brought about greater growth of the parent strain as shown in the section "Modification of the Medium" on p. 91.

The basal medium (double the final concentration) was prepared and 3.0 g. of vitamin-free casein hydrolysate added to 500 ml. The response to nicotinic acid in basal medium only was measured at the same time. The results of two experiments with strain 893 are given in Table 41 and the results for the first of the two experiments plotted in Fig. 13. The results show that the response at the lower concentrations of

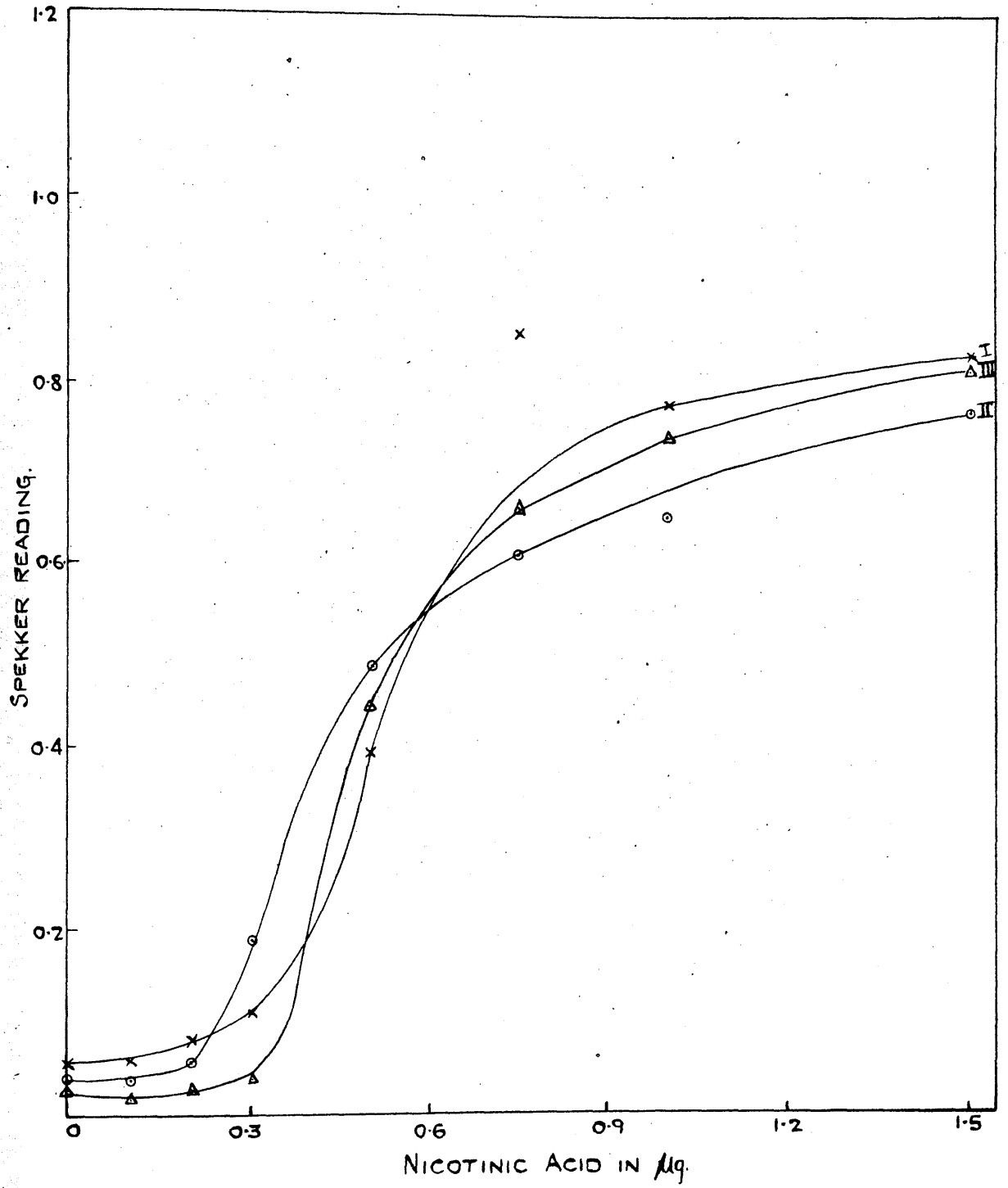


FIG. 12. VARIATIONS OF THE "LAG" IN GROWTH RATE OF STRAIN 893 WITH NICOTINIC ACID IN BASAL MEDIUM.

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS AT 37°C.

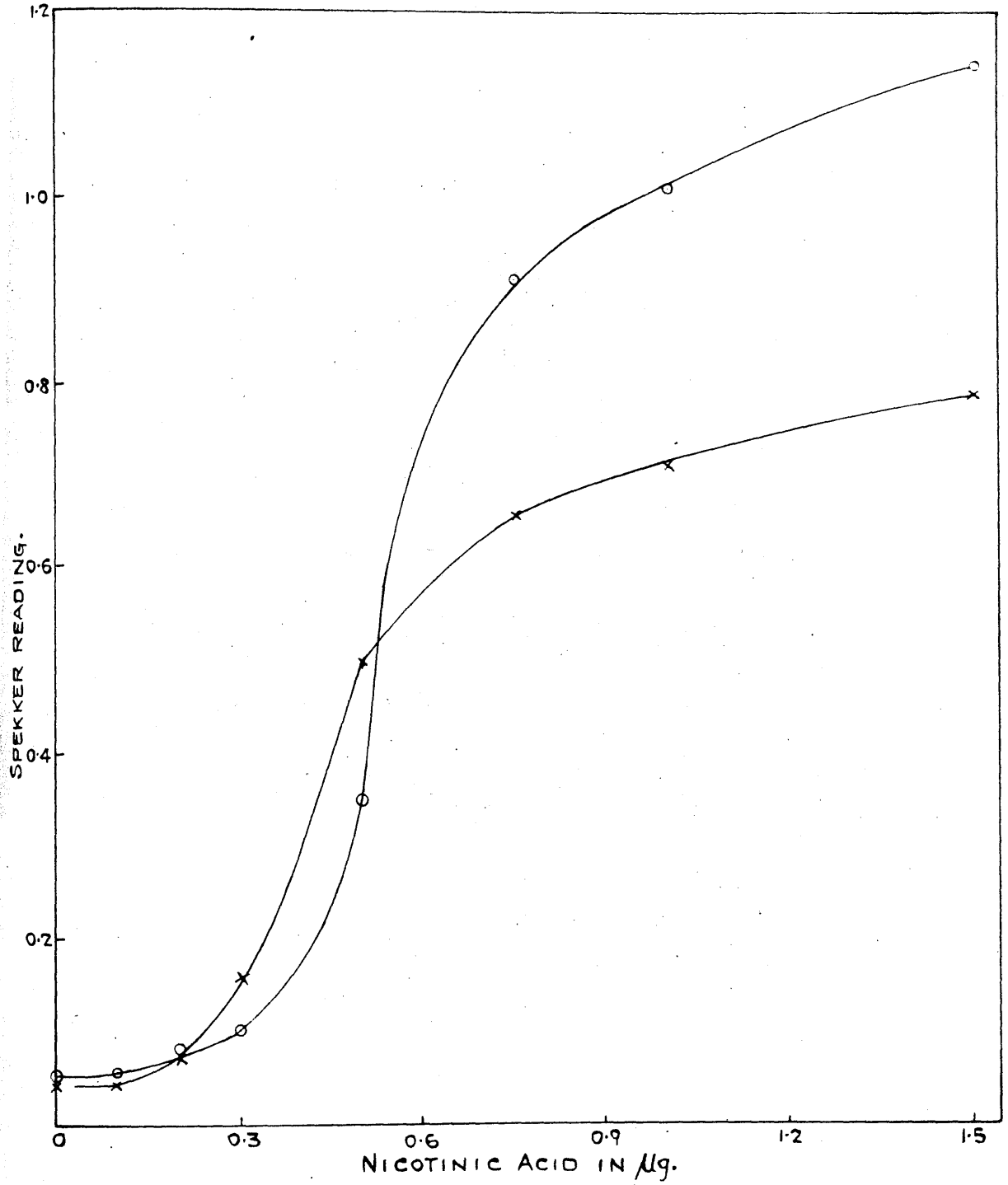


FIG. 13. DOSE-RESPONSE CURVE OF STRAIN 893 WITH NICOTINIC ACID IN BASAL MEDIUM WITH AND WITHOUT CASEIN HYDROLYSATE.

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS. AT 37°C.
×—× WITHOUT CASEIN HYDROLYSATE.
⊙—⊙ WITH CASEIN HYDROLYSATE.

TABLE 42.

Response to nicotinamide of strain 893 compared with response to nicotinic acid.

Turbidimetry after 17 hr. at 37°C. Spekker reading.

Concentration µg./tube.	Exp. 1+		Exp. 2+		Exp. 3=	
	Acid	Amide	Acid	Amide	Acid	Amide
0	0.038	0.018	0.031	0.004	0.016	0.006
0.1	0.057	0.147	0.018	0.154	0.021	0.247
0.2	0.075	0.257	0.027	0.279	0.042	0.454
0.3	0.119	0.356	0.041	0.375	0.058	0.635
0.5	0.443	0.543	0.442	0.561	0.126	0.819
0.75	0.609	0.658	0.660	0.709	0.386	0.985
1.0	0.718	0.749	0.737	0.777	0.878	1.070
1.5	0.779	0.813	0.810	0.851	1.083	1.145

+ Basal medium. = Basal medium plus casein hydrolysate.

nicotinic acid was less in medium containing casein hydrolysate than in basal medium alone. Therefore although the maximum response to the higher concentrations of nicotinic acid was greater with casein hydrolysate its addition was not useful in the removal of the "lag" in growth.

Response to nicotinamide of strain 893.

The response to 0.1, 0.2, 0.3, 0.5, 0.75, 1.0 and 1.5 µg. nicotinamide of strain 893 was determined turbidimetrically and compared with the response to nicotinic acid. Three experiments were conducted, the first two in basal medium only, the third in basal medium containing vitamin-free casein hydrolysate (3 g. in 500 ml. of double strength basal medium). The results are given in Table 42 and the results of Experiments 2 and 3 plotted in Fig. 14.

As shown previously in original medium there was no "lag" in growth with nicotinamide.

With concentrations above 0.3 µg. of nicotinic acid the growth curve in the basal medium was parallel to those for amide. In the presence of casein hydrolysate, growth with nicotinamide was greater at all concentrations than in the basal medium alone while growth with nicotinic acid at concentrations up to 0.9 µg. was less and at higher concentrations greater than in basal medium.

This "lag" in growth with nicotinic acid would

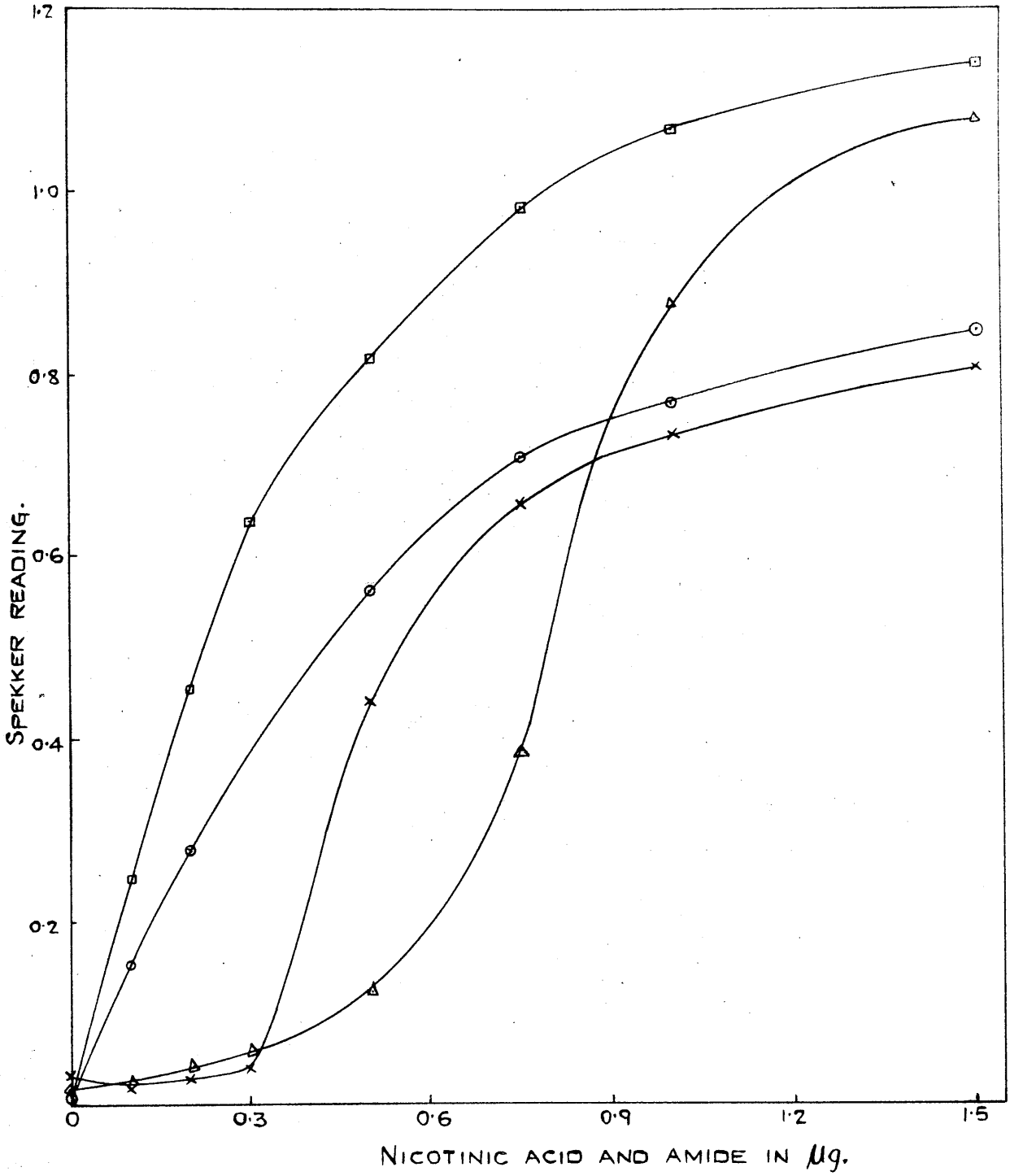


FIG. 14. DOSE-RESPONSE CURVE OF STRAIN 893 WITH NICOTINAMIDE AND NICOTINIC ACID IN BASAL MEDIUM WITH AND WITHOUT CASEIN HYOROLYSATE

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS. AT 37°C.
×——× NICOTINIC ACID WITHOUT CASEIN HYOROLYSATE.
⊙——⊙ NICOTINAMIDE " " "
△——△ NICOTINIC ACID WITH " " "
⊡——⊡ NICOTIHAMIDE " " "

TABLE 43.

Response to nicotinic acid of 893 with differing amounts of inoculum.

Turbidimetry after 17 hr. at 37°C. Spekker reading.

Nicotinic acid µg./tube.	Dilutions of inoculum.			
	1+	1:2	1:5	1:10
0	0.049	0.039	0.049	0.056
0.1	0.050	0.041	0.040	0.051
0.2	0.059	0.046	0.044	0.045
0.3	0.129	0.066	0.064	0.075
0.5	0.431	0.168	0.153	0.128
0.75	0.712	0.549	0.445	0.435
1.0	0.804	0.702	0.578	0.540
1.5	0.853	0.805	0.655	0.622

+The Spekker reading of the turbidity of dilution 1 was 0.165.

not permit the use of these two strains, 893 and $3A_3$ in microbiological assay of nicotinic acid as at low concentrations growth was not proportional to the amount of nicotinic acid present. It is possible that under different conditions - larger inoculum, different H-ion concentration, longer periods of incubation, etc.- this "lag" in growth might not occur. Therefore experiments were conducted to find out the effect of these factors on the response to nicotinic acid of the strain 893 in basal medium.

Effect of size of inoculum.

Two experiments with differing amounts of inoculum were conducted determining turbidimetrically the response to 0.1, 0.2, 0.3, 0.5, 0.75, 1.0 and 1.5 µg. nicotinic acid. Four different dilutions of a uniform suspension of the inoculum were prepared and turbidity measured on the Spekker absorptiometer. 0.05 ml. of each dilution was used as inoculum. The results of one experiment, given in Table 43 and plotted in Fig. 15, showed that the "lag" at low concentrations and the response at higher concentrations depended on the size of the inoculum. In both experiments it was not possible completely to overcome the "lag" in growth with the inocula employed. These experiments show that one of the factors favouring the production of maximum response to nicotinic acid was a large inoculum.

Effect of H-ion concentration.

The effect of H-ion concentration was studied by

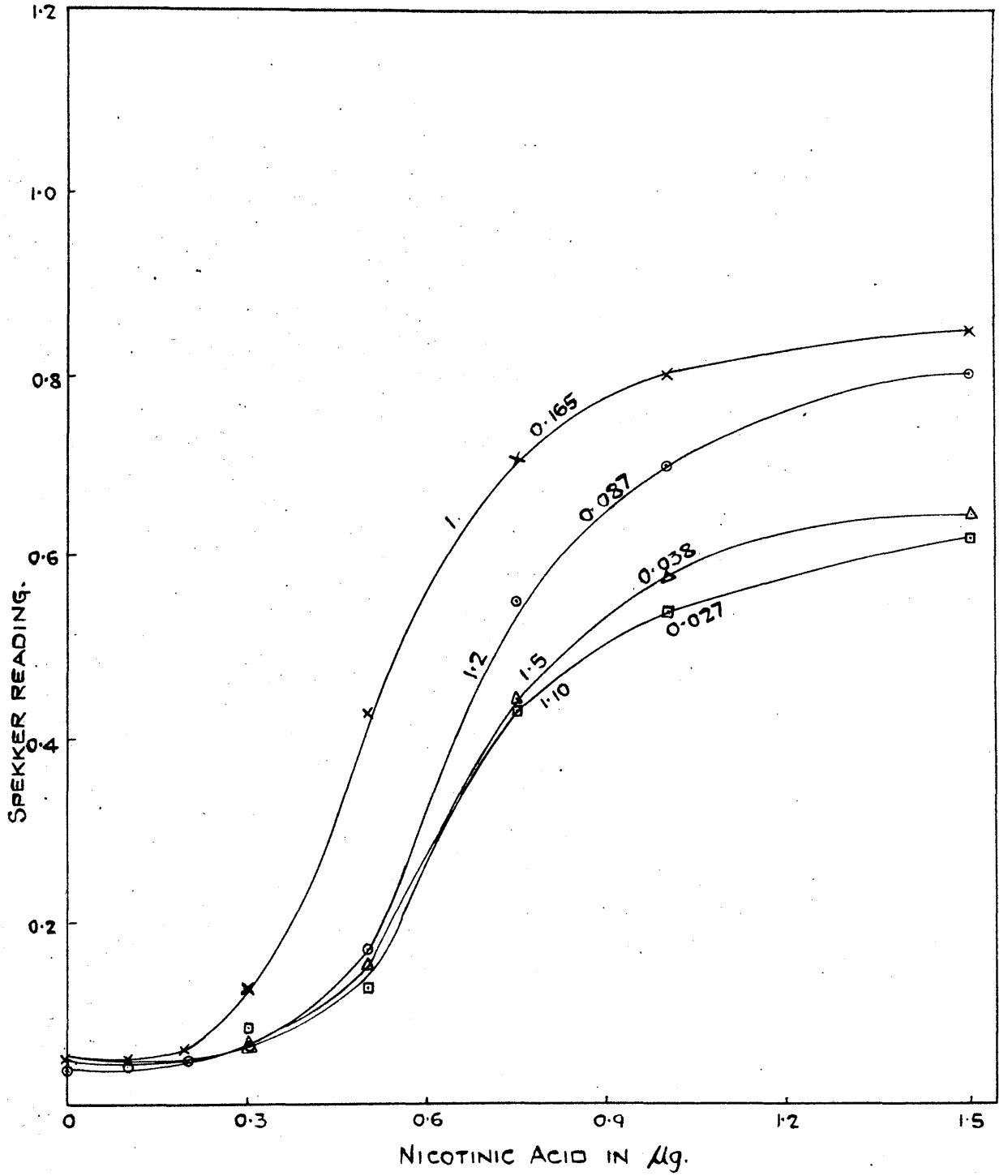


FIG. 15. EFFECT OF SIZE OF INOCULUM ON GROWTH RATE OF STRAIN 893 WITH NICOTINIC ACID.

DOSE-RESPONSE CURVES. TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS AT 37°C.

FIGURES DENOTE DILUTION AND SPEKKER READING OF THE TURBIDITY OF THE INOCULUM.

TABLE 44.

Effect of H-ion concentrations on the response to Nicotinic acid of strain 893.

Turbidimetry after 17 hr. at 37°C. Spekker reading.

Nicotinic acid µg./tube.	pH.			
	5.5	6.1	6.4	7.1
0	0.011	0.019	0.021	0.041
0.1	0.156	0.149	0.061	0.040
0.2	0.266	0.267	0.241	0.041
0.3	0.329	0.385	0.356	0.077
0.5	0.449	0.591	0.550	0.418
0.75	0.443	0.625	0.590	0.644
1.0	0.468	0.641	0.661	0.726
1.5	0.499	0,649	0.688	0.799

determining turbidimetrically the response to graded doses of nicotinic acid of strain 893 at pH 5.5, 6.1, 6.4 and 7.1. The results, Table 44, Fig. 16, showed that at pH 5.5 and 6.1 there was no "lag" period in growth. The cells thus seemed to utilise nicotinic acid for their metabolic processes more efficiently at an acid pH than when the pH was more nearly neutral.

The response to higher concentrations of nicotinic acid was greater at neutral pH than at acid pH. Repetition of this experiment at pH 6.0, 6.5, 6.9 and 7.3 confirmed that increase in the "lag" period occurred with increasing pH. The dose-response obtained at pH 6.0 by the technique normally used in this work was compared with that obtained at the same pH when the nicotinic acid was sterilised separately and added to the sterilised medium aseptically. There was no difference in the response from that when the nicotinic acid was added to the medium before sterilisation.

These results showed that an acid pH was one of the factors which gave an increasing response with increasing concentrations of nicotinic acid till the maximum response was attained and that this acid pH should be controlled within narrow limits.

Effect of period of incubation.

In the present work the measurement of growth response in terms of turbidity was normally made after

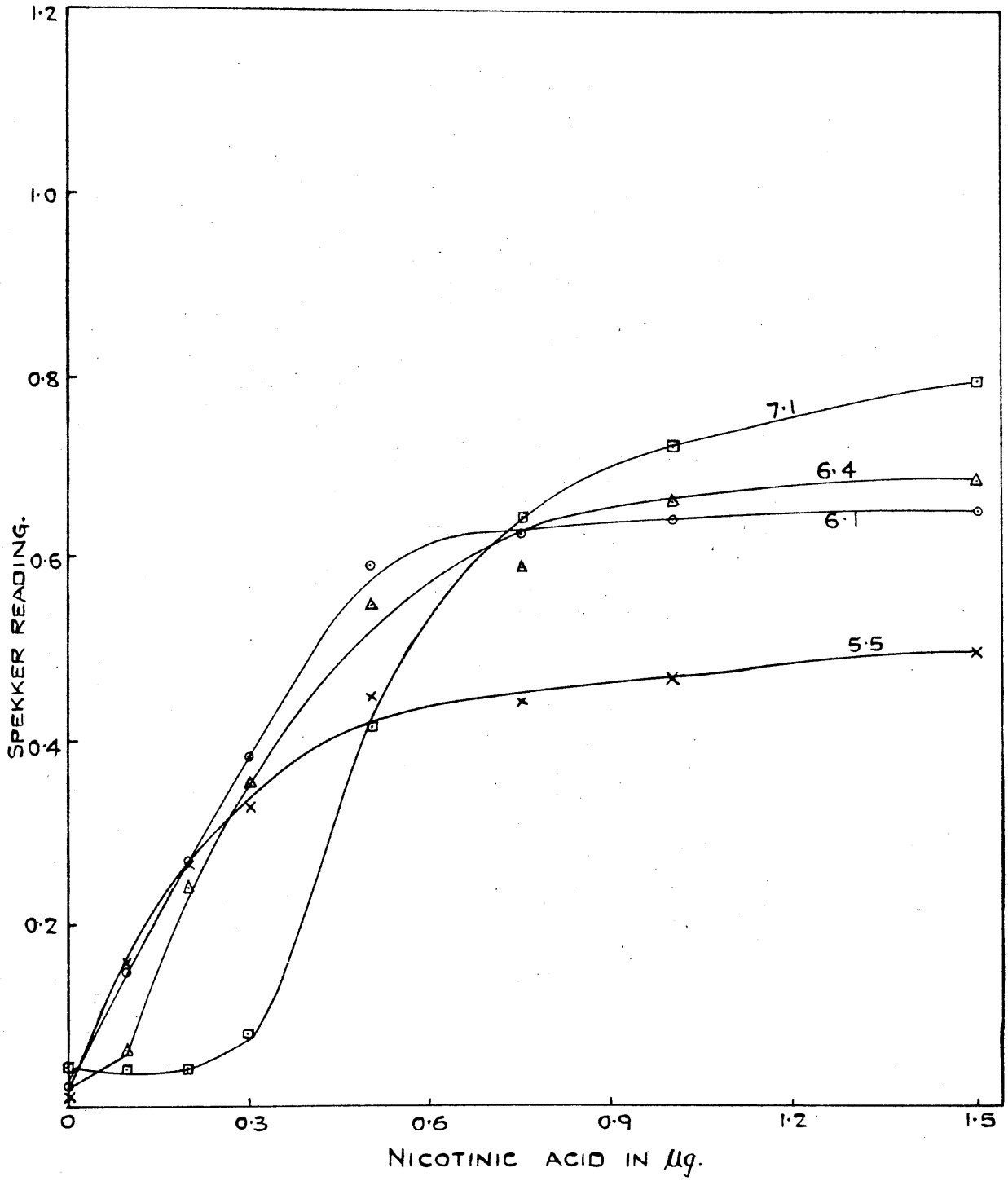


FIG. 16. EFFECT OF H-ION CONCENTRATION ON THE GROWTH RATE OF STRAIN 893 WITH NICOTINIC ACID.

DOSE-RESPONSE CURVES. TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS AT 37°C.

FIGURES DENOTE PH.

TABLE 45.

Effect of period of incubation on response to nicotinic acid of strain 893.

Turbidimetry. Incubation at 37°C. Spekker reading.

Nicotinic acid µg./tube.	Exp. 1.		Exp. 2.	
	17 hr.	41 hr.	17 hr.	48 hr.
0	0.030	0.026	0.031	0.004
0.1	0.033	0.136	0.018	0.003
0.2	0.074	0.246	0.027	0.119
0.3	0.159	0.325	0.041	0.207
0.5	0.581	0.529	0.442	0.463
0.75	0.713	0.755	0.660	0.769
1.0	0.782	0.829	0.737	0.844
1.5	0.932	1.115	0.810	0.895

incubation for 17 hr. at 37°C. The effect of longer periods of growth on the response to 0.1, 0.2, 0.3, 0.5, 0.75, 1.0 and 1.5 µg. nicotinic acid in media at approximately pH 7.0 was determined after incubation at 37°C. for 17, 41 and 48 hr. The results are given in Table 45 and Fig. 17. The "lag" in growth response after incubation for 41 or 48 hr. was considerably reduced or completely removed depending on the magnitude of the "lag" in any particular determination. Thus in Experiment 1 where the "lag" in response at 17 hr. occurred up to about 0.3 µg. nicotinic acid, this was completely removed after 41 hr. In a second experiment after incubation for 17 hr. the "lag" occurred up to 0.35 µg. and even after 48 hr. the "lag" still occurred up to 0.1 µg. nicotinic acid.

Thus, the "lag" in growth response to low concentrations of nicotinic acid was more or less completely removed by an acid pH of about 6.0 and by increasing the period of incubation. The associated disadvantages were (1) a lower maximum response to the higher concentrations of nicotinic acid at the lower pH, and (2) the much longer time required to obtain the result of the assay.

The fact that no lag was shown in acidimetric determinations of growth response (p.١٠٥) would be explained by the longer periods of incubation normally allowed before the acidimetric determinations were made.

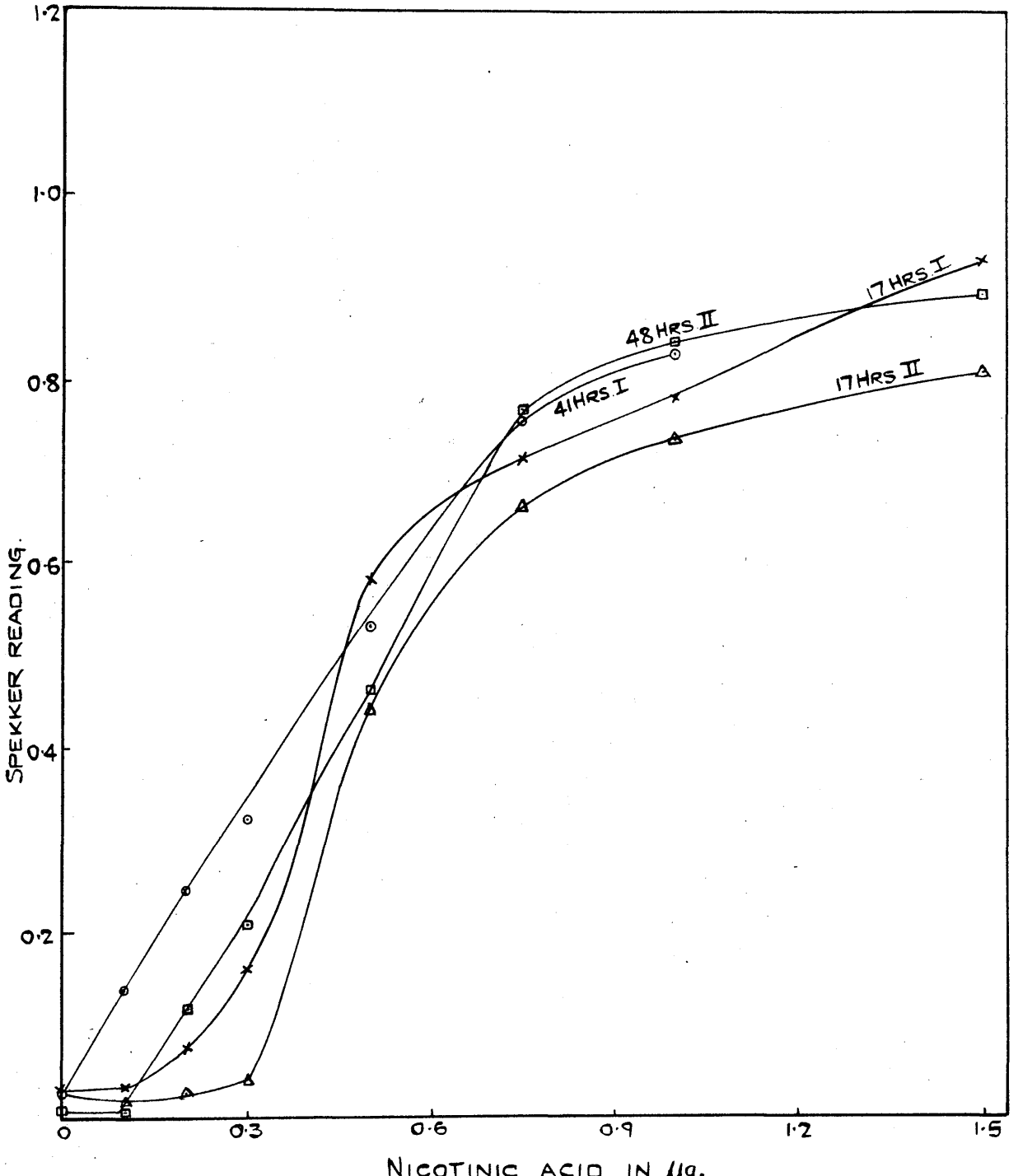


FIG. 17. EFFECT OF PERIOD OF INCUBATION ON GROWTH RATE OF STRAIN 893 WITH NICOTINIC ACID.

DOSE-RESPONSE CURVES. TURBIDIMETRIC MEASUREMENTS

FIGURES DENOTE EXPERIMENT NO. AND THE PERIOD OF INCUBATION.

TABLE 46.

Response to nicotinic acid of strains 893 and $3A_3$ in basal medium containing nicotinamide.

0.05 µg. amide per tube. Turbidimetry after 17 hr. at 37°C. Spekker reading.

Nicotinic acid µg./tube.	893		$3A_3$	
	Medium.		Medium.	
	Basal	+amide	Basal	+amide.
0	0.041	0.110	0.027	0.118
0.1	0.040	0.162	0.026	0.189
0.2	0.041	0.244	0.051	0.303
0.3	0.077	0.352	0.116	0.408
0.5	0.418	0.550	0.515	0.577
0.75	0.644	0.662	0.653	0.683
1.0	0.726	0.743	0.726	0.742
1.5	0.799	0.801	0.794	0.811

Since the "lag" period did not occur in response to nicotinamide, the possibility of eliminating the "lag" by the inclusion of a "minimal" amount of nicotinamide in the basal medium was investigated.

Effect of addition of a minimal amount of nicotinamide to the basal medium.

5 µg. of nicotinamide were added to 500 ml. of double strength basal medium giving a final concentration of 0.05 µg. per assay tube. The response to graded doses of nicotinic acid in the basal medium with and without the addition of nicotinamide was determined turbidimetrically. Two experiments were conducted with 893 and one with $3A_3$. The results of one experiment with each are given in Table 46.

Fig. 18 shows that the "lag" in growth was removed in the presence of nicotinamide indicating that once growth of the strains began they responded regularly to all concentrations of nicotinic acid.

Response to quinolinic acid of strain 893 and $3A_3$.

Both strains 893 and $3A_3$ had previously been shown (p.48) to respond qualitatively to quinolinic acid. The quantitative response to this acid has therefore been determined.

Response to quinolinic acid in basal medium was determined turbidimetrically. The doses first used were equimolecular to 0.1, 0.2, 0.3, 0.5, 0.75, 1.0 and 1.5 µg. nicotinic acid. No growth was obtained at

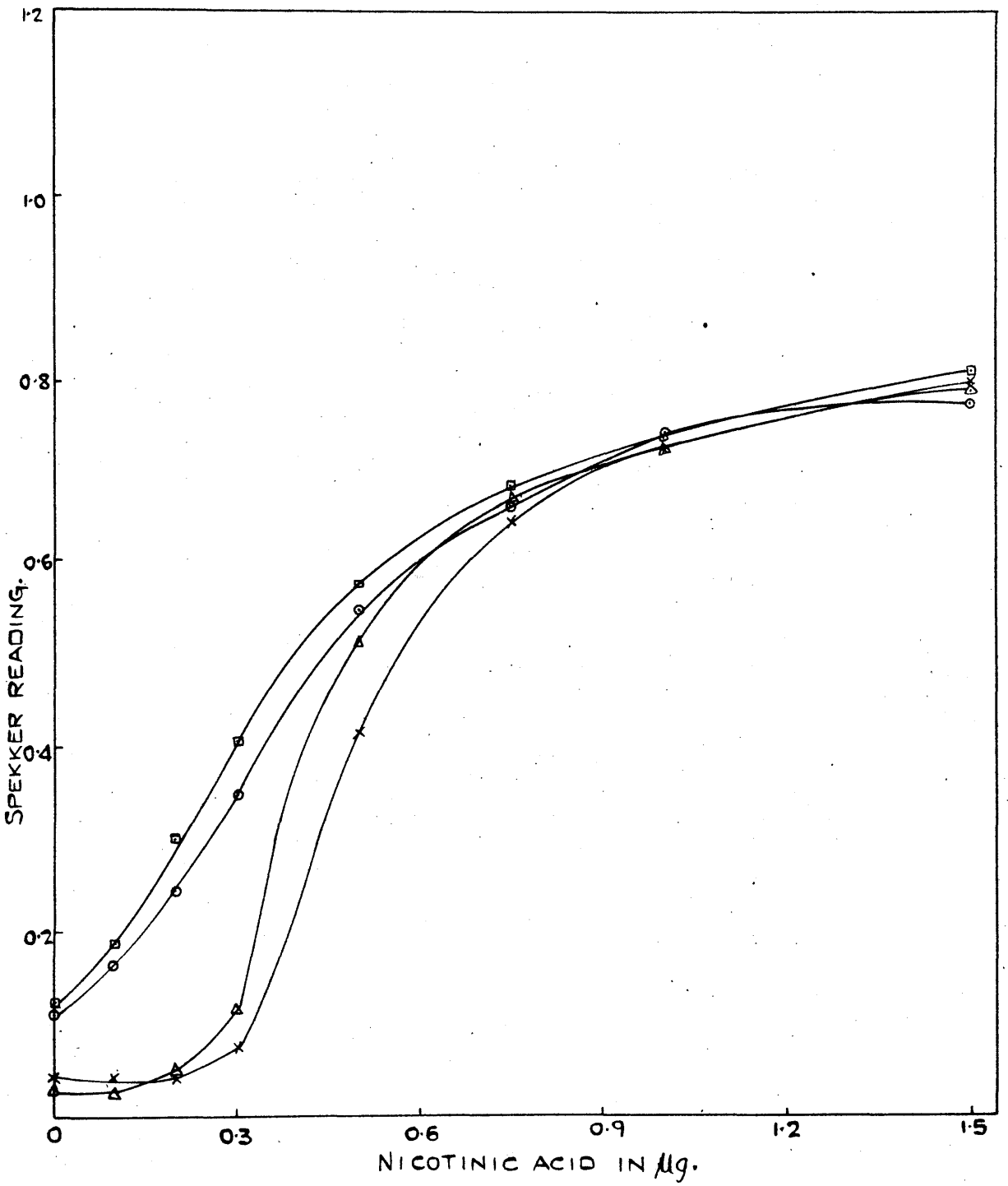


FIG. 18. DOSE-RESPONSE CURVE OF STRAIN 893 AND 3A3 WITH NICOTINIC ACID IN BASAL MEDIUM WITH AND WITHOUT NICOTINAMIDE.

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS AT 37° C.

×—× STRAIN 893, WITHOUT AMIDE.
⊙—⊙ " " WITH AMIDE.
△—△ STRAIN 3A3, WITHOUT AMIDE.
⊡—⊡ " " WITH AMIDE.

TABLE 47.

Response to quinolinic acid and nicotinic acid of strains 893 and 3A3.

Turbidimetry after 17 hr. at 37°C. Spekker reading.

893				3A3			
Nicotinic acid		Quinolinic acid		Nicotinic acid		Quinolinic acid	
µg/tube	Response	µg/tube	Response	µg/tube	Response	µg/tube	Response
0	0.034	0	0.032	0	0.027	0	0.019
0.1	0.035	10	0.029	0.1	0.050	10	0.026
0.2	0.054	20	0.029	0.2	0.137	20	0.027
0.3	0.189	50	0.028	0.3	0.293	50	0.051
0.5	0.487	100	0.046	0.5	0.608	100	0.123
0.75	0.606	300	0.520	0.75	0.653	300	0.595
1.0	0.650	500	0.624	1.0	0.747	500	0.740
1.5	0.766	1000	0.707	1.5	0.840	1000	0.752

these dose levels. Higher doses equimolecular to 10, 20, 50, 100, 300, 500 and 1000 µg. nicotinic acid were then used. Appreciable growth was obtained only with about 50 to 100 µg. The response to quinolinic acid compared with the response to nicotinic acid is given in Table 47, Fig. 19. The results showed that quinolinic acid gave a similar response to nicotinic acid only when present in about 1000 times the quantity of nicotinic acid.

Discussion.

That the first step in the utilisation of nicotinic acid is the synthesis of nicotinamide has been suggested by the fact that by far the most important role played by nicotinic acid is in relation to pyridine nucleotides where it is present as the amide.

The "lag" in growth response with low concentrations of nicotinic acid but not with the amide suggests that under such conditions the bacterial cell cannot synthesise nicotinamide from the acid as rapidly as with higher concentrations of nicotinic acid. The same phenomenon has been observed by Bonner and Beadle (1946) with mutant strains of Neurospora and by Roepke, Libby and Small (1944) with nicotinic-less mutants of *E. coli*.

The experiments presented above show that this "lag" in growth is removed or decreased by acid pH,

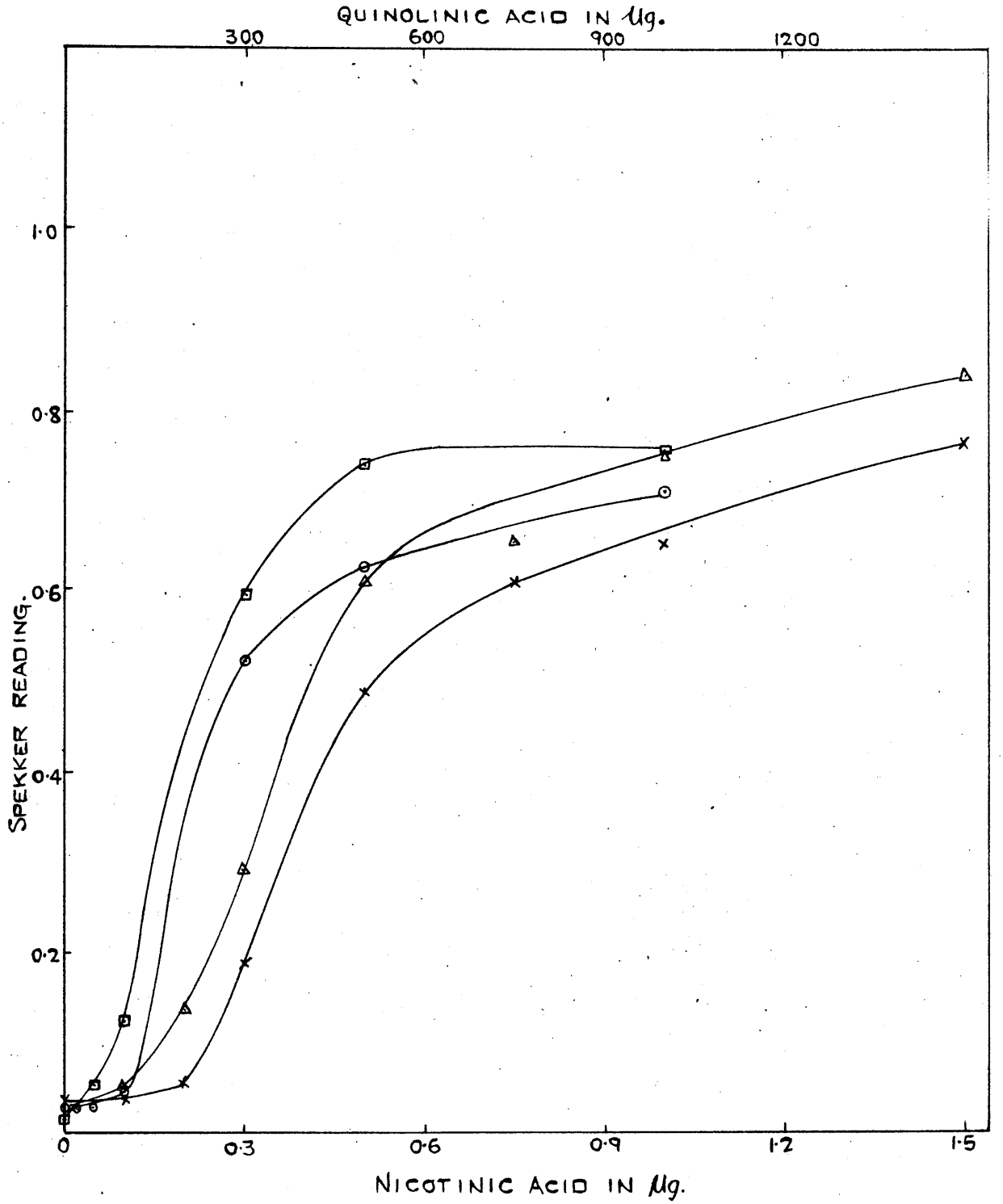


FIG. 19. DOSE-RESPONSE CURVES OF STRAINS 893 AND 3A3 WITH QUINOLINIC ACID COMPARED WITH NICOTINIC ACID.

TURBIDIMETRIC MEASUREMENTS AFTER 17 HRS AT 37°C.

× — × STRAIN 893, NICOTINIC ACID.
⊙ — ⊙ " " QUINOLINIC ACID.
△ — △ STRAIN 3A3, NICOTINIC ACID.
⊡ — ⊡ " " QUINOLINIC ACID.

longer periods of incubation and larger inoculum and that the dose-response curves under these conditions tended to approach that of nicotinamide. The effect of pH had been observed also by Bonner and Beadle (1946) and Roepke, Libby and Small (1944). They found a close correlation between activity of nicotinic acid at certain pH and dissociation values. The dissociation of nicotinic acid decreased with decrease in pH. They concluded that it was only the undissociated acid which could enter the cell and be utilised for metabolic purposes. But the fact that experiments with larger inocula and with minimal amounts of amide in basal medium do not give a "lag" in growth shows that once the growth starts nicotinic acid can be used rapidly enough even at neutral pH.

A similar phenomenon of inability to use acid instead of amide was observed in the utilisation of glutamic acid by Lactobacillus arabinosus (Lyman, Kuiken, Blotter and Hale, 1944; 1945). Lyman and co-workers suggested that glutamic acid must be converted to some other substance before it could be utilised and glutamine was suggested as the most obvious possibility. They found that addition of glutamine to the medium permitted growth to begin and that the growth curve then obtained with glutamic acid rose regularly with increasing concentration in a similar way to the results which have been presented here and which were obtained when nicotinamide was added to the

basal medium. In an independent investigation, Hac, Snell and Williams (1945) showed that the "lag" in growth decreased with increase in inoculum and also with longer periods of incubation and with more acid pH just as shown here for the nicotinic-less mutants. These authors concluded that glutamic acid must be converted to glutamine before it could be utilised. A similar conclusion that nicotinic acid must be converted to nicotinamide before utilisation follows from the present work.

Conclusion.

(1) The growth with nicotinic acid at low concentration is not proportional to the amount of nicotinic acid present. This resulting "lag" is dependent on the size of the inoculum, pH, and period of incubation.

(2) The growth with nicotinamide is proportional to the amount present at all concentrations, till the maximum response was obtained.

(3) The growth with nicotinic acid when a small amount of amide is present in the medium is the same as with the amide itself, giving a quantitative response at all concentrations up to 1.5 µg.

(4) The mutant strains grow with quinolinic acid but require about 1000 times as much quinolinic as nicotinic acid to bring about appreciable growth.

(5) These strains may be used for the microbiological

assay of nicotinic acid provided a minimal amount of amide is added to the medium and the pH of the medium and the size of the inoculum are strictly controlled.

Summary.

The effect of modifications of the original basal medium on the growth and acid production by Bact. aerogenes was studied. The effect of addition of various sources of nitrogen, purines and buffering agents separately and in association was investigated.

A final modified "minimal" medium was adopted which contained three times the phosphate and ammonium sulphate present in the original "minimal" medium.

Using this modification as the basal medium the response of the histidine-less mutants 506, 297 and $9B_2$ to graded doses of histidine was determined and was found to be quantitative.

The response of the nicotinic-less mutants 893 and $3A_3$ to nicotinic acid, nicotinamide and quinolinic acid was studied. The dose-growth response curve with nicotinic acid but not with nicotinamide showed a "lag" in growth response with the lower concentrations of the test substance.

The period of the "lag" was reduced by increase in the amount of inoculum and by longer incubation. The "lag" was completely removed by the inclusion of a minimal amount of nicotinamide in the basal medium or by adjustment of the medium to an acid pH.

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ACKNOWLEDGMENT.

My grateful thanks are due to Dr E.R. Dawson of the Distillers Company Ltd. for providing me with facilities to gain experience in the use of micro-biological methods of assay and also to all the other members of his staff in his laboratories at Great Burgh, Epsom. My thanks are also due to the following for the supply of certain chemicals: Prof. H.A. Krebs, Department of Biochemistry, The University, Sheffield, for glutamine; Roche Products Ltd. for thymine; Dr E.R. Dawson for 4-methyl-5-β-hydroxy ethyl thiazole and 2-methyl-4-amino-5-ethoxy-methyl pyrimidine and Dr H. McIlwain, Department of Biochemistry, The University, Sheffield, for 2, 4, 5-triamino-6-hydroxy pyrimidine sulphate.